A new safeguard eliminates T cell receptor gene-modified auto-reactive T cells after adoptive therapy

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To my parents

Contents

Summary

The adoptive transfer of T cell receptor (TCR) gene-modified T lymphocytes into patients is associated with potential risk factors. First, auto-immunity may occur if a tumor-associated antigen is targeted on normal tissue, if TCR chain mispairing leads to the formation of an auto-reactive receptor or if an otherwise anergic endogenous receptor specific for an auto-antigen becomes activated. Second, retroviral integration could lead to malignant transformation of the T cell. Therefore, it is essential to have the possibility to deplete the transferred T cells *in vivo* in case of severe side effects. The available safety modalities such as suicide gene/prodrug systems or cell surface proteins that are targeted by specific antibodies comprise disadvantages rendering them less feasible for the application in adoptive therapy with TCR gene-modified T cells.

In this thesis, a new safeguard has been developed which is based on a TCR-intrinsic depletion mechanism and can eliminate auto-reactive TCR-redirected T cells. By introducing a 10 amino acid-long sequence of the human c-myc protein (myc-tag) into the murine OT-I and P14 TCRs or the human gp100 TCR it was possible to deplete TCR-expressing T cells *in vitro* and *in vivo* with a myc-specific antibody. Depending on the antibody isotype, either complement lysis or antibody-dependent cell-mediated cytotoxicity could be induced. The T cells maintained equal function compared to cells expressing the wild-type receptor as shown by MHC-tetramer binding and cytokine secretion.

Importantly, the *in vivo* depletion of adoptively transferred T cells could prevent disease in an auto-immune mouse model. Here, splenocytes transduced with a myc-tagged OT-I TCR were injected into RIP-mOVA mice which express the OT-I-specific antigen ovalbumin in the β-islet cells of the pancreas. Destruction of these cells by the adoptively transferred T cells led to severe diabetes in untreated mice. Animals which received a myc-specific antibody after T cell transfer remained healthy and showed no increase in blood glucose levels.

The developed safeguard allows termination of adoptive therapy in case of severe side-effects. The strategy is superior to previous ones as it relies on a TCR-intrinsic mechanism which does not require introduction of an additional gene. Safety is not hampered by loss or low expression of the transgene and immunogenicity in humans is unlikely.

Zusammenfassung

Der adoptive Transfer von T-Zellrezeptor- (TZR-) modifizierten T Zellen ist mit potentiellen Risiken verbunden. Erstens können Autoimmunreaktionen auftreten, wenn Tumor-assoziierte Antigene auf normalem Gewebe erkannt werden, Fehlpaarung der TZR-Ketten zur Bildung eines autoreaktiven Rezeptors führen oder ein sonst anerger endogener Rezeptor aktiviert wird, der ein Autoantigen erkennt. Zweitens besteht das Risiko der malignen Transformation der Zelle durch Insertionsmutagenese des Retrovirusvektors. Daher ist es notwendig, die transferierten T Zellen im Falle schwerer Nebenwirkungen eliminieren zu können. Verfügbare Sicherheitsmechanismen wie Suizidgene oder Oberflächenmoleküle, die von spezifischen Antikörpern erkannt werden, sind für adoptive Therapie mit TZR-modifizierten T Zellen aufgrund vieler Nachteile ungeeignet.

In dieser Arbeit wurde ein neuer Sicherheitsansatz entwickelt, der auf einem TZR-intrinsischen Depletionsmechanismus beruht und autoreaktive, TZR-veränderte T Zellen eliminieren kann. Durch Einfügen einer 10 Aminosäure-langen Sequenz des humanen c-myc Proteins (myc-tag) in murine (OT-I, P14) und humane (gp100) TZRs konnten TZR-exprimierende T Zellen *in vitro* und *in vivo* mittels eines myc-spezifischen Antikörpers depletiert werden. Abhängig vom Isotyp des Antikörpers konnte Komplement-abhängige Lyse oder Antikörper-vermittelte zelluläre Zytotoxizität gezeigt werden. Die T Zellen behielten vergleichbare Funktionalität hinsichtlich Antigenerkennung und Zytokinsekretion wie Zellen, die den Wild-Typ Rezeptor exprimierten.

Die *in vivo* Depletion adoptiv transferierter T Zellen verhinderte lethalen Diabetes in einem Mausversuch. Im verwendeten Modell wurden Splenozyten, die mit einem myc-getagten OT-I TZR transduziert wurden, in RIP-mOVA Mäuse injiziert, die in den Inselzellen des Pankreas das OT-I-spezifische Antigen Ovalbumin exprimieren. Zerstörung der Inselzellen durch die transferierten T-Zellen induzierte lethalen Diabetes in unbehandelten Mäusen. Tiere, denen ein myc-spezifischer Antikörper verabreicht wurde, zeigten keine Symptome. Dieser neuartige Sicherheitsmechanismus erlaubt es, adoptive T Zelltherapie abzubrechen, falls schwere Nebenwirkungen auftreten. Im Gegensatz zu früheren Strategien beruht diese auf einem TZR-intrinsischen Mechanismus, bei dem kein zusätzliches Gen eingebaut werden muss. Die Sicherheit des Ansatzes wird durch Verlust oder Herunterregulierung des Transgens nicht beeinflusst und Immunogenität im Menschen ist unwahrscheinlich.

1 Introduction

1.1 The T cell receptor complex

The structure of the T lymphocyte antigen receptor resembles in many features that of an antibody Fab fragment. The T cell receptor (TCR) is a heterodimer consisting of either a TCRα-chain and a TCRβ-chain (for $\alpha\beta$ T cells) or a TCRγ-chain and a TCRδ-chain (for $\gamma\delta$ T cells) which are covalently linked by a disulfide bond. The majority of T cells express $\alpha\beta$ TCRs. $\gamma\delta$ T cells only make up about 5% of the peripheral T lymphocyte population and their precise role in the immune system and in tumor immunology is still under debate (reviewed in [1,2]). In this study, only the structure and function of $\alpha\beta$ TCRs will be discussed.

The TCR chains are expressed on the cell surface and each consists of an N-terminal variable part (V) that mediates the binding to antigen and the major histocompatibility complex (MHC), a constant region (C) that harbors the inter-molecular disulfide bond, a charged transmembrane domain and a short cytoplasmic tail that is involved in signal transduction (Figure 1A). The variable and constant parts are linked by a joining region (J) in the case of TCRα or a diversity region (D) and a joining region in the case of TCRβ. The existence of various V, J and D segments, which are recombined to one Cα segment or one of the two Cβ segments (Cβ1 and Cβ2) during T cell maturation, and the presence of three hypervariable complementarity determining regions (CDRs) in the variable domains of both TCR chains are the basis for the high diversity of TCRs. Each T cell expresses only one type of TCR and it was estimated that 10^8 – 10^9 different T cell clones circulate in any individual allowing the recognition of a multitude of pathogens.

Analysis of various crystallized TCR fragments (reviewed in [3,4,5]) revealed that each variable and constant domain display an immunoglobulin (Ig)-like "β-barrel" structure that consists of three to four anti-parallel β-sheets facing three similar sheets on the other side (Figure 1B). Only Cα diverges from this predicted Ig fold as its outer strands exhibit a random coil structure rather than β-sheets which may be the reason for an observed higher lability of the TCRα-chain [6,7,8].

On the cell surface, the TCR chains are expressed in complex with the CD3 subunits γ, δ, ε and ζ which associate to covalently linked CD3$\zeta\zeta$ homodimers and non-linked CD3$\gamma\varepsilon$ and CD3$\delta\varepsilon$ heterodimers [9]. The extracellular parts of CD3ε and CD3γ are predicted to adopt an

Ig-fold [10]; and the cytosolic region of all CD3 molecules harbor immuno-receptor tyrosine-based activation motifs (ITAMs) which can be phosphorylated and play a role in recruiting downstream signal transducers (Figure 1A). The stoichiometric composition of the TCR/CD3 complex is still controversially discussed. Some studies suggest that two TCR$\alpha\beta$ heterodimers are clustered with one CD3γ and CD3δ chain, two CD3ε chains and two CD3ζ chains (Figure 1C), others report that only one TCR$\alpha\beta$ molecule is involved (reviewed in [11,12]. However, there is compelling evidence that the formation of multivalent TCR/CD3 complexes upon MHC binding is required for full T cell activation [13,14,15].

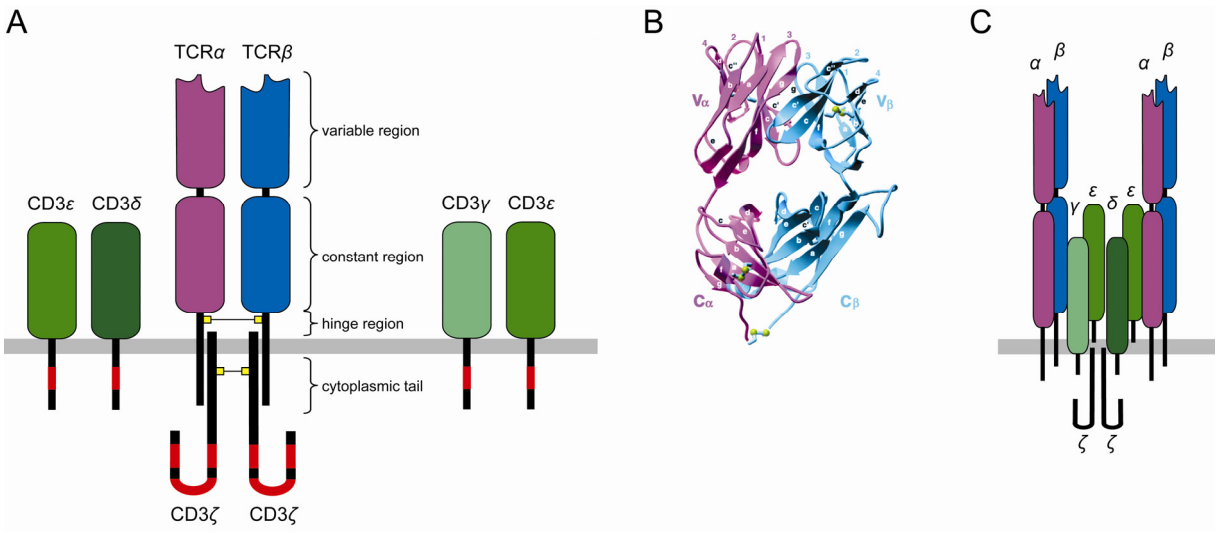

FIGURE 1: Schematic and crystal structure of the $\alpha\beta$ TCR complex. (A) A schematic drawing of a TCR, showing the TCRα-chain (purple) and the TCRβ-chain (blue) linked by a dilsulfide bond (yellow). Accessory CD3 molecules (green) mediate the signal transduction via ITAM motifs (red boxes). (B) Crystal structure of the extracellular part of the murine 2C TCR. The numbers in the V regions indicate the CDR loops. Adapted from [6]. (C) Presumed model of a TCR-CD3 cluster.

Several binding sites in the TCR chains have been identified that are crucial for the association with CD3 molecules. The FG loop in the Cβ domain – a large solvent-exposed protrusion – forms a cavity that is predicted to interact with one CD3ε subunit [16]. Also, critical amino acids in the extracellular and transmembrane parts of Cα and Cβ have been found that mediate the assembly with CD3ζ [17].

In contrast to antibodies, TCRs cannot recognize free antigens; instead they bind small peptide fragments that have been processed intracellularly and loaded on MHC molecules. Cytotoxic CD8-positive cells recognize antigens bound to MHC class I, whereas CD4-positive T

helper cells are specific for MHC class II-presented antigens. The peptide-MHC class I complexes are found on the surface of nearly every cell and represent the cell's whole proteome. Upon binding of the TCR to its cognate peptide-MHC complex, an immunological synapse between the T cell and the target cell is formed that leads to recruitment of specific molecules, e.g. adhesion proteins, to lipid rafts. The affinity of TCRs for their specific antigen is rather weak when compared to that of an antibody. Therefore, the interaction is further stabilized by coreceptors, such as CD4 and CD8 which bind to invariable parts of the MHC molecule.

1.2 T cell immuno-therapy for cancer

The role of the immune system in tumor development has been discussed for more than a century. Ehrlich postulated already in 1909 that the immune system might protect the host from cancer [18]. Some fifty years later, Thomas and Burnet formulated the cancer immunosurveillance hypothesis [19,20] saying that lymphocytes were eliminating continuously developing transformed cells. Though this hypothesis in the context of non-virally induced tumors is still highly debated today [21,22] numerous studies have demonstrated that cancer cells have an antigen pattern which is distinct from that of normal cells and that T cells can specifically recognize these tumor antigens (a list of known human tumor antigens can be found in [23]).

Although tumor-infiltrating lymphocytes (TILs) have been isolated from many human or animal malignancies, their presence alone is obviously not sufficient to reject an established tumor. One reason for this may be that many tumor antigens are auto-antigens that are aberrantly expressed in cancerous tissue, and therefore, may be only of low immunogenicity. T cells recognizing auto-antigens are usually deleted in the thymus during negative selection and auto-reactive lymphocytes that escape this central tolerance machinery are mainly of low affinity. Furthermore, it has been shown by Willimsky *et al.* that spontaneously developing tumors – although expressing a tumor-specific transplantation rejection antigen – can induce general T cell unresponsiveness and tolerance [24].

Nevertheless, the potential of T cells to infiltrate into tissues and to specifically recognize and destroy a cell presenting a foreign antigen make them a useful tool in anti-cancer therapy. The obstacles that inhibit naturally occurring tumor-specific lymphocytes might be overcome by

adoptive T cell transfer as this therapy provides the means to inject a large number of cells and to select or engineer T cells with a high affinity to one or several tumor antigens. Additionally, previous irradiation or chemotherapy of the patient can give the transferred T cells a proliferative advantage and augment the presentation of tumor antigens [25].

1.2.1 Transfer of unmodified T cells

The principle of adoptive T cell therapy is to isolate T cells either from the patient or an allogeneic donor, expand and activate them *in vitro* and transfer them back into the patient. To date, the most successful clinical application of T cell therapy is the treatment of chronic myeloid leukemia by MHC-matched allogeneic stem cell transfer [26,27]. It is assumed that donor T cells that are transferred along with the stem cells are the main effectors in preventing relapse. Most likely, disparities in minor histocompatibility antigens between donor and recipient are recognized and account for the elimination of residual malignant cells [28,29]. A hint to that is the observation that the anti-tumor effect is markedly decreased when T cells are depleted from the graft or when bone marrow derived from a genetically identical twin is transferred [30]. Still, an increased graft-versus-leukemia (GvL) effect also seems to correlate with increased incidence of graft-versus-host disease (GvHD) – an often lethal complication that occurs when the transplanted T cells attack normal tissue.

A second successful approach of transplanted T cells has been the treatment of virally-induced tumors which are frequent in immuno-suppressed patients, e.g. after stem cell or organ transplantation. Reinfused autologous or donor T cells effectively restored immunity against Epstein-Barr virus (EBV) [31,32,33,34] or cytomegalovirus (CMV) [35,36,37] and prevented lympho-proliferative disorders. A strong advantage in this type of application is the possibility to select, expand, clone and characterize antigen-specific T lymphocytes *in vitro* before transfer.

The translation of this approach to non-virally induced malignancies, however, was only of limited success (reviewed in [38]). This is most likely due to the lower immunogenicity of non-viral tumor antigens and the lower precursor frequency of TILs compared to virus-specific and allo-reactive T cells. One exception to this rule seems to be melanoma from which frequently large numbers of highly lytic TILs can be isolated [39]. Redefined culture and treatment conditions recently led to objective responses in 30 to 80% of melanoma patients in

recent clinical trials [40,41]. Still, given the required laborious and time-consuming *ex vivo* expansion of TILs, this therapy will only be available for a limited number of patients.

1.2.2 Transfer of gene-modified T cells

The genetic modification of T lymphocytes *in vitro* before adoptive transfer allows endowing these cells with enhanced properties or defined antigen receptors and might overcome many obstacles observed with unmodified T cells. Engineering T cells with a desired specificity by gene therapy has the advantage that (i) large numbers of therapeutic lymphocytes can be created in relatively short time compared to the long and cumbersome expansion of unmodified tumor-reactive T cells, (ii) specificities other than naturally occurring can be employed and (iii) treatment will also be feasible for patients from which TILs could not be isolated.

Chimeric antibody receptors (CARs)

CARs are fusion proteins of antibody Fv fragments and a TCR signaling domain [42] that recognize antigens independent from MHC. They have a high, antibody-like affinity and T cells transduced with CARs have been shown to kill tumor cells *in vitro* (reviewed in [43]). In a first clinical trial the *in vivo* function of CAR-transduced T cells specific for an epitope of carboxy-anhydrase-IX (CAIX), but also auto-immunity, was reported [44]. An important drawback in the use of CARs, though, is their restriction to cell surface tumor antigens which greatly limits their broad application. Furthermore, their high affinity bears the risk of auto-immunity when the tumor-antigen is also expressed on a normal cell.

T cell receptors

Another strategy to confer a T cell with tumor-specificity is the transfer of TCR genes. High-affinity TCRs for human tumor-associated antigens can either be isolated (i) from rare, highly reactive human TILs, (ii) by generation of peptide-specific, allo-reactive T cells [45], (iii) from HLA-transgenic mice immunized with human tumor antigens [46,47] or (iv) by *in vitro* mutagenesis using yeast or phage display techniques [48,49,50]. To date, a large panel of TCRs against viral and tumor antigens has been isolated which, when genetically transferred into a T cell, were able to redirect its specificity (reviewed in [51,52,53,54]). Genetic modification can either be transient through RNA electroporation or stable through the use of retrovi-

ral vectors. An initial clinical trial using PBLs transduced with a melanoma-antigen specific TCR has led to partial remission in some patients [55]. Still, many hurdles regarding the efficiency of TCR gene-modified T cells in tumor therapy have to be overcome. One is the high-level expression of the transgene, which can be hampered by competition with the endogenous TCR or by formation of mispaired TCR heterodimers composed of one endogenous and one transgenic TCR chain. Furthermore, the choice of the retroviral vector and composition of the transgene cassette have been shown to be important [7,56]. A second hurdle is the selection of the target antigen. In general, two groups of tumor antigens have been identified: (i) tumor-specific antigens (TSAs) that are only expressed on tumor tissue and are caused by random mutations of different cellular genes, and (ii) tumor-associated antigens (TAAs) that are found over-expressed in cancer cells, but also – albeit at a lower level – on normal tissue [23]. As TSAs are usually not shared between different patients, TAAs have been more extensively studied for use in immuno-therapy but bear the risk of therapy side effects when non-tumor tissue is damaged. Ideally, one would find a TCR specific for an antigen which is only expressed on the tumor, but shared between patients such as viral tumor antigens, *p53* or *ras* mutation hotspots, or newly created epitopes of fusion proteins such as *bcr-abl*. Finally, it has been shown that recognition of tumor cells alone is not sufficient to lead to tumor rejection due to the expansion of antigen-loss variants. To allow efficient elimination of the tumor, it is also necessary to target the tumor stroma [25,57,58]. In line with this, anti-angiogenic treatment or tumor site irradiation can augment T cell therapy response. While the first leads to shortage of blood supply of tumor cells, the latter promotes the cross-presentation of tumor-antigens by the stroma cells which thus become a target of adoptively transferred T cells.

1.3 Risk factors of adoptive T cell therapy

Despite first clinical responses achieved with genetically engineered T lymphocytes, several risk factors in the treatment of patients have to be considered. These can either results from the choice of the target antigen or the genetic manipulation of the T cell itself.

1.3.1 Recognition of tumor-associated antigens on self-tissue

Tumor-associated antigens are the most thoroughly studied targets in T cell based immunotherapy. The clinical application of a TCR which recognizes a TAA, however, bears the risk of

auto-immunity when the engineered T cells damage TAA-expressing non-tumor tissue. In some cases, mild auto-reactivity may well be tolerated as a side effect of the therapy. If, however, essential tissue is affected, it is desirable to have a possibility to eliminate the transferred T cells.

Adverse effects by adoptive T cell therapy have already been observed in some clinical trials and mouse models. In two independent studies, the destruction of melanocytes (vitiligo) and uveitis was seen in patients which had received melanoma antigen-specific T cells [40,41]. Vitiligo was also observed in tumor-bearing mice which received T cells specific for the melanoma antigen gp100 for treatment [59]. In another trial, patients with renal cell carcinoma were treated with PBLs genetically modified with a CAR specific for an epitope of CAIX. Here, liver cytotoxicity occurred due to specific reaction of the infused CAR-modified T cells with CAIX-expressing epithelial cells of the bile ducts and the study had to be discontinued [44]. Such auto-immune reactions in adoptive immuno-therapy most likely depend on the antigen level [60]. They can hardly be predicted, may vary from patient to patient and will become very important when TCR-redirected T cells are routinely used for therapy.

1.3.2 Formation of heterodimers by endogenous and transgenic TCR

It has been observed that the introduced TCR chains can individually form mixed heterodimers with the α- and β-chains of the endogenous TCR [61,62]. The extent of mispairing seems to both depend on the stability of the inter-chain interaction (preferential pairing), and on the intrinsic stability of the respective TCR chain and its ability to compete with the endogenous chain for export and accessory proteins (weak and strong TCRs [61]). The occurrence of mixed heterodimers has two principal consequences. First, it reduces the amount of correctly paired therapeutic TCR on the cell surface, and thereby most likely the functional reactivity of the engineered T cell. Second, if a polyclonal pool of T cells is TCR-modified, the specificity of the mispaired TCRs cannot be predicted and they might have auto-reactive capacity.

Several strategies have been tested that promote preferential pairing of the desired TCR chains. For example, the introduction of an additional disulfide bond [63,64], leucine zipper motifs [65] or the inverse exchange of an amino acid pair in the interface of the TCRα and TCRβ constant regions [66] stabilized the interaction of the transgenic TCR chains. Also, the

use of murine constant regions instead of human both enhanced the binding to human CD3 molecules and supported preferential pairing [67,68]. Willemsen *et al.* constructed chimeric single chain and two chain TCRs which did not pair with the endogenously expressed TCR chains [69]. Still, it remains unclear whether these amino acid modifications will be immunogenic and cause unwanted elimination of the transferred T cells by the host's immune system. Van der Veken *et al.* introduced a TCR into $\gamma\delta$ T cells, whose endogenous TCRs are unable to form heterodimers with $\alpha\beta$TCRs [70]. However, it is not known whether engineered $\gamma\delta$ T cells will exhibit the same anti-tumor function *in vivo* as $\alpha\beta$ T cells. Another option would be the use of RNA interference (RNAi) to down-regulate the endogenous TCR chains and the generation of a codon-modified transgenic TCR which is not influenced by the RNAi mechanism.

1.3.3 Activation of an endogenous auto-reactive TCR

Another safety concern is the possible activation of the endogenous TCR. While some data show that signaling through one TCR in dual-specific T cells is receptor-specific [71], others demonstrate that activation of the introduced TCR may also induce a response of the endogenous receptor [72]. Most likely the observed cross-activation varies in different model systems and cannot be predicted when polyclonal T cells are transduced. Although clonal deletion in the thymus eliminates the majority of T cells with high-affinity auto-reactive TCRs, low-affinity auto-reactive T lymphocytes escape central tolerance mechanisms [73,74]. If these T cells are transduced with a second TCR and become activated, they may react against self-tissue. Just as for the prevention of TCR heterodimers, the use of RNAi to modulate the expression of the endogenous TCR, or the pre-selection of T cells with a defined, non-auto-reactive specificity before transduction [75,76,77] might avoid this risk.

1.3.4 Insertional mutagenesis and transformation

It is assumed, that for prevention of relapse the long-term persistence of infused tumor-specific T cells is necessary. For this, stable TCR expression in the transduced T cells is essential. So far, all techniques that support stable expression require integration of the transgenic DNA into the host genome. Most effective delivery systems (e.g. retroviruses), however, allow only non-site-specific insertion which bears the risk of malignant transformation if the integration affects the expression of an oncogene. Until recently it has been assumed that integra-

tion occurs randomly. Considering the small proportion of gene-encoding regions in the human genome, it seemed very unlikely that oncogenes will be a target site for the vector.

Reports of serious adverse events in an otherwise successful clinical trial of gene therapy for X-linked severe combined immuno-deficiency (X-SCID), however, demonstrated retrovirus-induced lympho-proliferative disease in 4 of 9 treated patients in one study and 1 of 10 treated patients in a second study [78,79,80]. In four of the cases it was reported that retroviral integration activated expression of the proto-oncogene *lmo2* which finally led to the oncogenic transformation [79]. Since then, numerous studies analyzed retrovirus integration sites in human and murine cells and showed that integration occurs non-random and preferentially in the 5' region of transcriptionally active genes [81,82,83,84,85]. In the X-SCID trials, the fact that hematopoietic progenitor cells were transduced, which are probably more prone to transformation due to deregulation of expression, might have contributed to development of leukemia. In the case of retroviral transduction of T lymphocytes with a suicide gene, also preferential integration sites and deregulated expression profiles were found. This, however, seemed to have no consequences for the T cell biology and no clonal selection in patients was observed [86,87]. Whether genetic modification of T cells or hematopoietic stem cells using a TCR influences the expression pattern and leads to loss of polyclonality still needs to be analyzed. Besides, many attempts have been made to construct self-inactivating retroviral vectors, non-integrating vectors or vectors with site-specific integration and high efficiency [88,89,90] which in the future might overcome the obstacle of insertional mutagenesis.

In sum, immuno-therapy with TCR-modified T cells bears the potential risk of auto-immune side effects and malignant transformation of the T cell. Although approaches have been tested that avoid some specific risk factors, a general safety strategy allowing *in vivo* depletion the adoptively transferred T cells – thereby terminating the therapy – is desirable.

1.4 Potential safety mechanisms

In the case of severe side effects, several treatments allow the *in vivo* suppression of T cells. The administration of T cell-specific antibodies or corticosteroids can block auto-immune reactions, but also abolishes desired – e.g. anti-viral – immune responses. Therefore, a way to specifically eliminate the transferred lymphocytes is preferable. For this, several strategies have

been suggested. Among them are (i) suicide genes of viral or bacterial origin which drive the cell into apoptosis upon application of a prodrug, (ii) fusion proteins of cellular apoptosis-inducing molecules that cluster upon administration of a dimerizer drug, (iii) transgenic cell surface molecules which can be targeted by specific depleting antibodies, and (iv) MHC multimers that deliver cytotoxic substances to a specific T cell pool. The feasibility of these strategies in the context of therapy with TCR-modified T cells is discussed below.

1.4.1 Suicide gene/prodrug systems

The most thoroughly studied suicide gene so far is the thymidine kinase of Herpes Simplex Virus type 1 (HSV-TK). This enzyme plays a key role in the phosphorylation of thymidine to produce dTMP (Figure 2A). Unlike cellular kinases, the viral enzyme has very broad substrate specificity and also converts pyrimidine and purine analogs such as acyclovir and ganciclovir (GCV) (Figure 2B). The mono-phosphorylated forms of these analogs are further phosphorylated by cellular kinases and incorporated into nascent DNA leading to an arrest of DNA synthesis, DNA fragmentation and finally apoptosis of the cell.

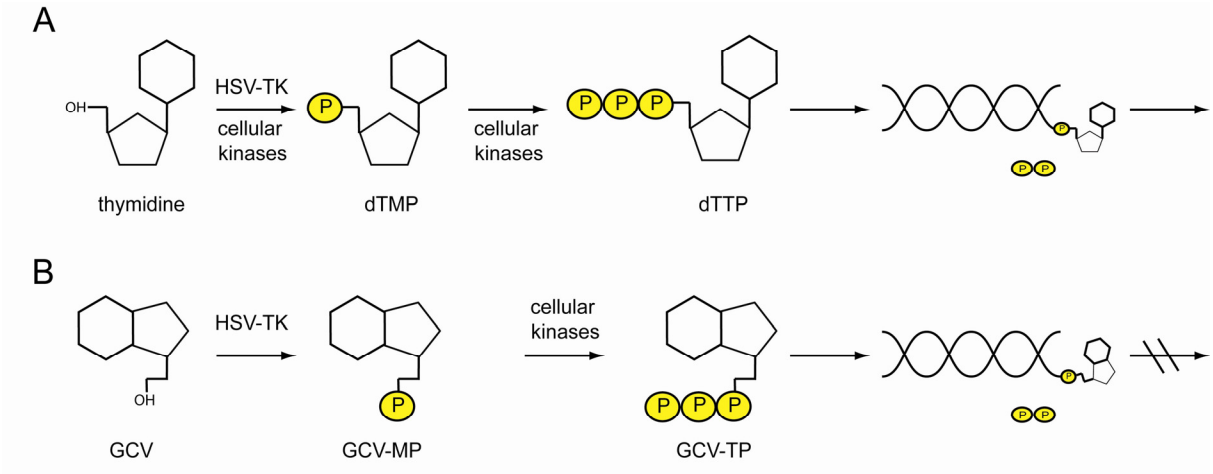

FIGURE 2: HSV-TK can phosphorylate thymidine and nucleotide analogs. (A) Thymidine is phosphorylated by cellular kinases or HSV-TK to thymidine-mono-phosphate (dTMP) and further to thymidine-tri-phosphate (dTTP) which can be incorporated into a replicating DNA strand. To the free OH- group, further nucleotides can be attached. (B) HSV-TK also phosphorylates nucleotide analogs like GCV, which in their tri-phosphorylated form are integrated in the newly synthesized DNA. As no further nucleotides can be attached to GCV, the replication process is discontinued.

HSV-TK in combination with the prodrug GCV has already been applied as a suicide gene in a variety of animal tumor models and for some human cancers (reviewed in [91,92,93]). More recently, it was suggested as a safety modality in patients who develop GvHD after donor lymphocyte infusion following T cell-depleted allogeneic hematopoietic cell transplantation (reviewed in [94,95,96]). Several clinical phase I-II trials have tested the safety and efficacy of this approach in humans [97,98,99,100]. Though application of HSV-TK in T lymphocytes was demonstrated to be safe and partially successful, the trials also revealed significant disadvantages of the strategy. Major limitations of the HSV-TK system are: (i) immunogenicity of the HSV-TK gene product resulting in immune responses and the elimination of transferred gene-modified T cells [99,101,102], (ii) transgene silencing or inhomogeneous transgene expression which leads in cells expressing low levels of HSV-TK to the development of ganciclovir-resistance [103,104], (iii) the prodrug ganciclovir cannot be used to treat upcoming viral infections (e.g. of CMV or EBV) in patients, and (iv) the restriction to proliferating cells which impedes elimination of slowly dividing T cells usually present in chronic GvHD. Apart from HSV-TK a number of other suicide gene of prokaryotic and eukaryotic origin are known. Their high expected immunogenicity, however, has restrained their use in therapy.

1.4.2 Apoptosis-inducing fusion genes and dimerizer prodrugs

An alternative to the HSV-TK-induced cell cycle arrest is to employ the cell's own mechanisms of apoptosis (illustrated in Figure 3A). Several different strategies attempted to construct fusion proteins in which a signaling domain of a protein involved in the apoptosis pathway (e.g. the DED domain of FADD, the FasR intracellular domain or a modified caspase 9) was linked to a FK506 binding protein (FKBP) [105,106,107,108]. Administration of a synthetic dimerizer prodrug leads to cross-linking of the FKBP domains and activates apoptosis (Figure 3B).

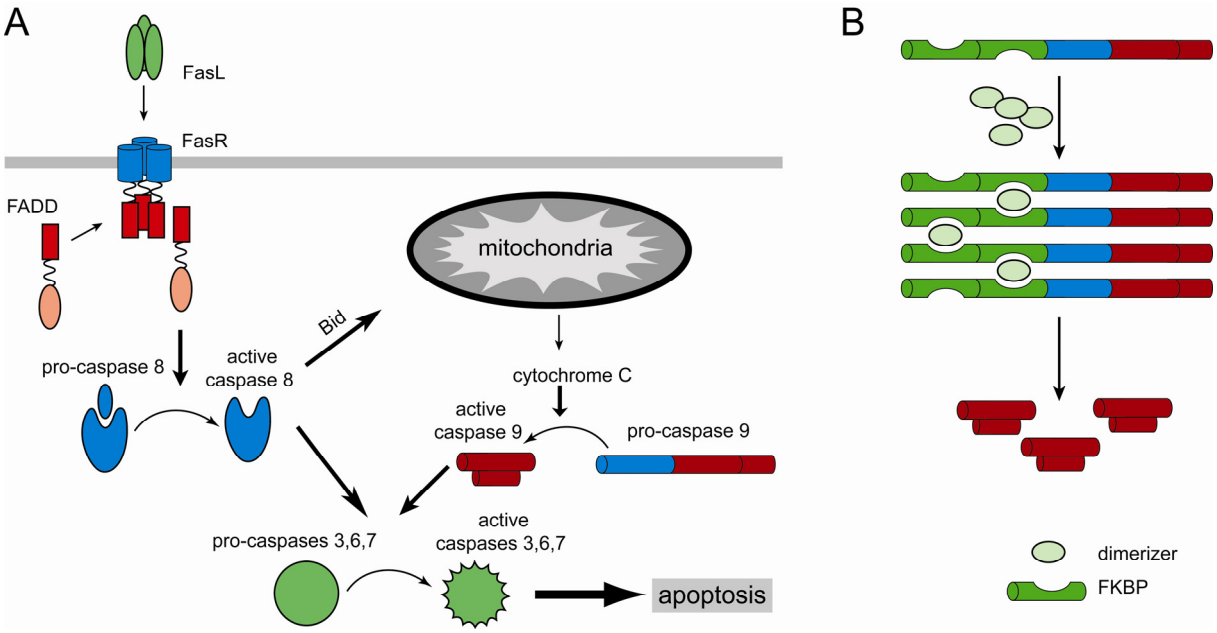

FIGURE 3: Mechanism of apoptosis induction by a caspase 9 fusion construct. (A) Pathway of Fas-induced apoptosis. By binding of Fas ligand (FasL) to the Fas receptor (FasR) the receptor molecules form a cluster bringing the death effector domains (DED, red boxes) in close proximity. This recruits FADD which subsequently leads to activation of downstream caspases. Cytochrome C release from mitochondria mediates the conversion of procaspase 9 into its activated form. (B) Structure of the caspase 9-FKBP fusion construct. Upon application of the prodrug the molecules multimerize which mimics the process of caspase 9 activation.

Compared to HSV-TK, apoptosis-inducing fusion genes comprise a lower risk of evoking an immune response against the transgene products as all of the fusion protein components are of human origin. Still, it cannot be excluded that an immunogenic peptide at the fusion sites of the several domains is generated, finally leading to an unwanted elimination of the transferred T cells. Besides, *in vitro* experiments with the caspase 9 transgene and a first *in vivo* study with the Fas-linked constructs in non-human primates revealed that a small proportion of T cells showed resistance to elimination and that in these cells the expression of the transgene was reduced [108,109,110]. Most likely all of the approaches depend on high levels of transgene expression and comprise the risk that silencing of the fusion construct abrogates the possibility of elimination. Furthermore, the use of apoptosis-inducing molecules like Fas or FADD that act upstream of many apoptosis inhibitors, such as bcl-2, bcl-x_L or c-FLIP, might lead to inhibition of T cell depletion when these molecules become up-regulated. This, however, is not the case for caspase 9, which is a late-stage apoptosis pathway molecule and maintains its function in T cells over-expressing anti-apoptotic proteins.

1.4.3 CD20 and CD20-depleting antibodies

CD20 is a cell surface molecule mainly expressed by B lymphocytes. It acts as a calcium channel in the cell membrane and is presumably involved in B cell activation. Its precise function, however, is still not known. Introna *et al.* and van Meerten *et al.* used retroviral or lentiviral vectors to transduce T lymphocytes with the CD20 cDNA [111,112,113]. They could show that CD20 can be expressed on a high level on primary human T cells and T cell lines and that the transduced cells can be enriched via CD20 surface expression. Furthermore they demonstrated that CD20-modified T cells can be depleted by incubation with a CD20-specific antibody and complement factors.

One advantage of this strategy is the availability of a CD20-specific antibody which is well characterized and approved for clinical use. This monoclonal antibody (Rituximab) has originally been developed for the treatment of CD20-positive B cell lymphomas, but is now also used in some B cell-associated auto-immune diseases. The large clinical experience with Rituximab would ease the implementation of this safety approach in a clinical setting. Compared to the above described safety approaches, modification of T cells with CD20 has the advantage that the molecule is very unlikely to be immunogenic as the entire transgene is of human origin.

Still, administration of the antibody would also lead to the unwanted elimination of the patient's B cells. Furthermore, resistance of lymphoma cells to Rituximab treatment has been reported [114] and the efficacy of depletion has been demonstrated to depend on the level of transgene expression [115]. As survival of CD20-modified T lymphocytes will most likely not depend on functional CD20 expression, mutations in the transgene or silencing of the transgene may occur, rendering the T cells resistant to elimination. An additional concern is that the presence of the B cell-specific CD20 molecule in T lymphocytes might alter their phenotype. Although there is a small subset of T cells expressing CD20 naturally [116], the function of the molecule in these cells remains unclear. Serafini *et al.* analyzed CD20-modified T cells *in vitro* with respect to antigen-specific and allo-induced cytokine release, chemotaxis and the expression of activation markers [117]. They observed no differences between mock-transduced and CD20-transduced T lymphocytes. However, it cannot be excluded that *in vivo* the CD20 molecule might affect the homing behavior or function of the T cell.

1.4.4 Cytotoxic tetramers

MHC multimers are broadly used for the detection and quantification of specific T cell populations *in vitro* and *in vivo*. By coupling a cytotoxic agent to the MHC molecule, it is also possible to deplete T cells of certain specificity. Yuan *et al.* fused MHC tetramers to the short-range, alpha-emitting isotype ^{225}Ac which specifically killed their cognate T lymphocytes in culture [118]. In a different approach, Hess *et al.* coupled MHC tetramers to the type I ribosome-inactivating protein saporin [119]. When these tetramers bind to the specific TCR, the entire complex becomes internalized into the cell where saporin inhibits protein synthesis finally leading to apoptosis. When injected into mice, the saporin-MHC tetramers were able to deplete about 75% of the target T cells. However, in this experiment also mild, transient cytotoxic side effects like loss of body weight and hepatopathy were observed. A similar strategy was used by Casares *et al.* who constructed MHC class II chimeras coupled to doxorubicin, an antimitogenic drug [120].

Although the clonal deletion of T cells by cytotoxic tetramers seems advantageous, this approach also comprises several drawbacks. First, MHC tetramers coupled to cytotoxic agents have low structural stability. Though they might be useful in depleting T cells *ex vivo* (e.g. from grafts), their *in vivo* application might be hampered by their short half-life and their inability to infiltrate poorly vascularized tissues. Second, the injection of cytotoxic substances into patients might lead to unwanted bystander effects and their specificity needs to be carefully evaluated. Third, the *in vivo* administration of MHC multimers has been shown to modulate immune function. Some studies found that injection of soluble MHC induced antigen-specific T cell unresponsiveness [121,122], while others revealed that it has a T cell-activating effect [123,124]. Finally, the production and safety testing of individualized suicide tetramers for each tumor antigen-specific TCR would be very cost-intensive.

In sum, all of the described safety mechanisms comprise several limitations rendering them inappropriate for T cell therapy with TCR gene transfer. Combining two different safety approaches may overcome some of the disadvantages [125,126]; however, this very much complicates the generation of TCR-modified T cells. Apart from depleting tetramers, all strategies require introduction of at least one additional gene into T cells. Retroviral vectors – the most commonly used system to stably transduce T cells – only have a limited transgene capacity. Considering the size of the TCRα- and TCRβ-chain genes it is unlikely that vectors that carry an additional gene can efficiently transduce T cells. Hence, PBLs will necessarily have to be

independently transduced with a TCR and a second gene-encoding vector. This increases the number of retroviral integrations into the host cell genome, and thus the risk of insertional mutagenesis [127]. Also, the purification and analysis steps needed to ensure that all TCR-redirected cells express the safety modality will prolong the *in vitro* culture time and decrease their functionality [128]. Table 1 summarizes the advantages and disadvantages of each approach.

Safety approach	Advantages	Drawbacks
HSV-TK / GCV	- clinically approved prodrug - safe and partially efficient in clinical trials	- immunogenicity - slow response - gene silencing or deletion - no GCV treatment possible - dependence on expression level, purification, insertion of additional gene
Apoptosis-inducing fusion proteins	- fast response - low expected immunogenicity	- possible cytotoxicity - dependence on expression level, purification, insertion of additional gene
CD20 / CD20-mAb	- clinically approved antibody - no immunogenicity expected - fast response	- unwanted elimination of B cells - possible change of phenotype - dependence on expression level, purification, insertion of additional gene
Cytotoxic tetramers	- not dependent on expression level, purification, insertion of additional gene	- structural instability - individualized, cost-intensive production - possible cytotoxic bystander effects - immunomodulation

TABLE 1: Safety modalities for adoptive T cell transfer.

1.5 Outline of this thesis

The aim of this thesis was to develop a method for the specific depletion of adoptively transferred TCR-gene modified T cells. Such safeguard has to meet several criteria: (i) it should not interfere with TCR function; (ii) it should be specific, efficient and rapid; and (iii) the implementation in a clinical setting should be feasible.

With respect to this, the objective was to select a short amino acid sequence (tag) and introduce it into the TCR structure so that it can be recognized by a tag-specific antibody. *In vivo*, the binding of a depleting tag-specific antibody would then lead to a specific elimination of a T cell expressing the tag-modified TCR.

For this purpose, the myc-tag – a peptide derived from the human c-myc protein – was inserted into various positions of the model murine TCR P14 gene. The wild-type and myc-tag-modified TCRs were cloned into retroviral vectors which were used to transduce murine T lymphocytes. The properties of the expressed TCR molecules were analyzed *in vitro*:

- First, it was tested whether introducing a myc-tag allowed expression and assembly of the TCR on the cell surface and if so, whether the expression level was influenced by the myc-tag.
- Second, it needed to be investigated whether the myc-tag was inserted in a conformation in which it could be bound by a myc-specific antibody.
- Third, complement-mediated depletion assays were performed to see whether the binding of a myc-specific antibody was able to induce depletion of the T cells *in vitro*.
- Fourth, the function of T cells transduced with a myc-modified TCR – including antigen binding and cytokine secretion upon antigen stimulus – was analyzed and compared to T cells transduced with a wild-type TCR.

Having found a position for myc-tag insertion which allowed functional expression of the TCR and supported lysis by a myc-specific antibody, the objective was to test the universality of this approach when applied to other TCRs. Therefore, a second murine TCR (OT-I, specific for an ovalbumin-derived peptide) and one human TCR (gp100, specific for a common melanoma antigen) were modified with a myc-tag in the same position. Murine and human T cells transduced with these TCRs were analyzed as described above.

Finally, the application of the safeguard was investigated *in vivo*. For this, T cells transduced with a myc-tag-modified OT-I TCR were transferred into RIP-mOVA mice which express ovalbumin in the pancreatic islet cells. As without treatment the mice succumb to auto-immune diabetes, it was analyzed whether application of a myc-specific antibody allowed the specific depletion of the auto-reactive OT-I T cells and hence a rescue of the animals from the otherwise lethal disease.

2 Material and Methods

2.1 Material

2.1.1 Oligonucleotides

Oligonucleotides were obtained as lyophilized stocks from MWG Biotech or TIB MOLBIOL.

Oligonucleotides for insertion of a myc-tag into different positions of a TCR

TCR	Position	Primer	Sequence
P14*α*		fwd	TTTGCGGCCGCAGTCTAGGAGGAATGGACAAG
		rev	GCCCTGTACATCAACTGGACCACAGCCTCAG
	AN	myc1	TCCTCCTCGCTGATCAACTTCTGCTCGCCATTCACCCCGGCTAGATGTAGG
		myc2	AGAAGTTGATCAGCGAGGAGGACCTGCAGCAGAAGGAGAAACATGACCAGC
	CS	myc1	ATCTTCTTCAGAAATAAGCTTTTGCTCGGAGTCAAAGTCGGTGAACAGGCAGAG GGT
		myc2	CAAAAGCTTATTTCTGAAGAAGATCTGGGAACGTTCATCACTGACAAAACTGTG
P14*β*		fwd	TTTGCGGCCGCCTGAGAGGAAGCATGTCTAAC
		rev	GGGCCGTGTACATCAGGAATTTTTTTTCTTGACC
	BN	myc1	ATCCTCCTCAGAGATCAGTTTTTGCTCAGCCTCCATGTGTTTTGTTCCCAG
		myc2	CAAAAACTGATCTCTGAGGAGGATCTGGCAGTCACCCAAAGTCCAAGAAGCAA
	L1	myc1	CTCCTCTGAAATCAGTTTTTGCTCAAGCCCATGGAACTGCACTTGGCAGCGG
		myc2	GAGCAAAAACTGATTTCAGAGGAGGATCTGCCCAAACCTGTCACACAGAACATC
	L2	myc1	CTCCTCGCTGATCAGCTTCTGCTCCTCTGACAGCCCATGGAACTGCACTTGG
		myc2	CAGAAGCTGATCAGCGAGGAGGACCTGCCTGTCACACAGAACATTAGTGCCGAG
	L3	myc1	TCCTCCTCGCTGATCAGCTTCTGCTCGTCCTCCTCTGACAGCCCATGGAACT
		myc2	GAAGCTGATCAGCGAGGAGGACCTGACACAGAACATTAGTGCAGAGGC
	XL	myc1	TCCTCCTCGCTAATCAGCTTCTGCTCTGGCCACTTGTCCTCCTCTGACAGCCCA
		myc2	AGAAGCTGATTAGCGAGGAGGACCTGGAAGGCTCACCCAAACCTGTCACACA
OT-I*α*		fwd	TTTGCGGCCGCAGTCTAGGAGGAATGGACAAG
		rev	GCCCTGTACATCAACTGGACCACAGCCTCAG

	AN	myc1	TCCTCCTCGCTGATCAACTTCTGCTCGCCATTCACCCCGGCTAGATGTAGG
		myc2	AGAAGTTGATCAGCGAGGAGGACTTGCAGCAGCAGGAGAAACGTGACCAGC
gp100*α*		fwd	CTGCGGCCGCCATGGTGAAGATCCGG
		rev	CCTGTACATCAGCTGGACCACAGCCGCA
	AN	myc1	CTCCTCGCTGATCAGCTTCTGCTCGGCACTTACACAGCTTAACTGGAGCCAC
		myc2	AAGCTGATCAGCGAGGAGGACCTGGCCAAAAATGAAGTGGAGCAGAGT

Oligonucleotides for insertion of a second myc-tag

Primer	Sequence
M1	GATCAGCGAGGAGGACCTGGAGCAGAAGTT
M2	GATCAACTTCTGCTCCAGGTCCTCCTCGCT

Oligonucleotides for cloning of P2A-linked TCR chains

TCR	Primer	Sequence
P14	fwd	TTTGCGGCCGCAGTCTAGGAGGAATGGACAAG
	P2Arev	GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCCG CTTCCACTGGACCACAGCCTCAGCGTCATG
	P2Afwd	GGCAGCGGAGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCG GTCCCATGTCTAACACTGCCTTCCCTGAC
	rev	CCGGAATTCTCAGGAATTTTTTTTCTTGACC
OT-I	fwd	TTTGCGGCCGCAGTCTAGGAGGAATGGACAAG
	P2Arev	GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCCG CTTCCACTGGACCACAGCCTCAGCGTCATG
	P2Afwd	AACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGTCTAACA CTGTCCTCGCTGATTCT
	rev	GGGCCGTGTACATCAGGAATTTTTTTTCTTGACC

Oligonucleotides for sequencing of genes in the MP71 vector

Primer	Sequence
fwd	CAGCATCGTTCTGTGTTGTCT
rev	CACCTGAACTAGTAATTACATATCC

2.1.2 Plasmids and retroviral vectors

Name	Description
pcDNA3.1gag/pol	Eukaryotic expression vector encoding murine leukemia virus (MLV) *gag* and *pol* genes; a gift from C. Baum (Hannover, Germany)
pALF-10A1GaV	Eukaryotic expression vector encoding murine leukemia virus (MLV) *env* gene 10A1; [129]
MP71-P14α(X)	Retroviral vector MP71 [56] expressing the P14 TCRα-chain (with a myc-tag in position X)
MP71-P14β(X)	Retroviral vector MP71 expressing the P14 TCRβ-chain (with a myc-tag in position X)
MP71-OT-Iα(X)	Retroviral vector MP71 expressing the OT-I TCRα-chain (with a myc-tag in position X)
MP71-OT-Iβ	Retroviral vector MP71 expressing the OT-I TCRβ-chain
MP71-gp100α(X)	Retroviral vector MP71 expressing the gp100 TCRα-chain (with a myc-tag in position X)
MP71-gp100β(X)	Retroviral vector MP71 expressing the gp100 TCRβ-chain (with a myc-tag in position X)
MP71-P14α(X)-P2A-P14β	Retroviral vector MP71 expressing the P14 TCRα- (with a myc-tag in position X) and TCRβ-chain linked by a P2A peptide
MP71-OT-Iα(X)-P2A-OT-Iβ	Retroviral vector MP71 expressing the P14 TCRα- (with a myc-tag in position X) and TCRβ-chain linked by a P2A peptide

2.1.3 Peptides and tetramers

Ova-peptide (SIINFEKL), gp33-peptide (KAVYNFATM) and gp100-peptide (IMDQVPFSV) were purchased as HPLC-purified products from Biosyntan. PE- or APC-labeled tetramers were used to stain gp100 TCR (Immunomics), P14 TCR (Immunotech) and OT-I TCR (D. Busch, Munich, Germany).

2.1.4 Mice strains

C57BL/6J mice were purchased from Charles River. B and T cell-deficient Rag-1$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}) mice were obtained from The Jackson Laboratory. RIP-mOVA mice (a gift from T. Brocker, Munich, Germany) express chicken ovalbumin under control of the rat insulin promoter in the β-islet cells of the pancreas [130]. All mice were housed and bred at the ani-

mal facility of the Max Delbrück Center for Molecular Medicine, Berlin, Germany. Animal experiments were approved by the responsible institution and performed according to national and regional regulations.

2.1.5 Cell types and media

Cell type	Source	Description	Medium
293T	American Type Culture Collection (ATCC) CRL-11268	Human embryonic epithelial cells	DMEM + GlutaMAX I (GIBCO), 10% active fetal calf serum (FCS, Biochrom), 100 IU/ml Pen/Strep (GIBCO)
Plat-E	[131]	Ecotropic packaging cell line based on 293T, stable expression of MLV *gag-pol* and *env* genes	DMEM + GlutaMAX I, 10% heat-inactivated FCS, 100 IU/ml Pen/Strep, 1 µg/ml puromycin (Sigma), 10 µg/ml blasticidin (MP Biomedicals)
58	[132]	TCRα- and TCRβ-negative variant of BW5147 cell line (murine T cell lymphoma)	T cell medium (RPMI 1640 + GlutaMAX I, 10% heat-inactivated FCS, 1 mM HEPES pH 7.25 (Sigma), 100 IU/ml Pen/Strep)
B3Z	[133]	Hybridoma of a lacZ-inducible derivative of BW5147 and ova-specific T cell clone B3	T cell medium
Jurkat76	[134]	TCR-deficient derivative of J.RT3-T3.5 Jurkat cells	T cell medium
RPMI 8866	G. Trinchieri, Philadelphia, USA	Human lymphoblastoid cell line	T cell medium
T2	ATCC CRL-1992, P. Cresswell, New Haven, USA	Human TAP-deficient hybridoma	T cell medium, 50 µM mercaptoethanol (Sigma)
T2-K^b	H. Schreiber, Chicago, USA	T2 transfected with H2- K^b	DMEM + GlutaMAX I, 5% heat-inactivated FCS, 100 IU/ml Pen/Strep, 1 mg/ml G418 (Invitrogen)
Murine splenocytes	Spleens of C57BL/6J mice		CMM medium (RPMI 1640 + GlutaMAX I, 10% heat-inactivated PAN-FCS (PAN Biotech), 1 mM HEPES, 1% Na-Pyruvat (GIBCO), 100 IU/ml Pen/Strep, 50 µM mercaptoethanol)
Human PBLs and NK cells	Healthy donors		PAN T cell medium (RPMI 1640 + GlutaMAX I, 10% heat-inactivated PAN-FCS, 1 mM HEPES pH 7.25, 100 IU/ml Pen/Strep, 10-100 IU/ml rhIL-2 (Chiron))

2.1.6 Antibodies

Specificity	Conjugate	Clone	Isotype	Host	Application	Source
Human CD28		CD28.2	IgG_1	Mouse	PBL stimulation	BD
Human CD3		OKT3	IgG_{2a}	Mouse	PBL stimulation	CILAG
Human CD3ε	Fluorescein isothiocyanat (FITC)	SK7	IgG_1	Mouse	Flow cytometry (FC)	BD
Human CD56	Allophyco-cyanin (APC)	B159	IgG_1	Mouse	FC	BD
Human TCR vβ8	Phyco-erythrin (PE)	56C5.2	IgG_{2a}	Mouse	FC	Immunotech
Mouse CD16/32		2.4G2	IgG_{2b}	Rat	Fc receptor block	BD
Mouse CD28		37.51	IgG_2	Syrian hamster	Splenocyte stimu-lation	BD
Mouse CD3		145-2C11	IgG_1	Armenian hamster	Splenocyte stimu-lation	BD
Mouse CD3e	FITC	145-2C11	IgG_1	Armenian hamster	FC	BD
Mouse CD3ε	APC	145-2C11	IgG_1	Armenian hamster	FC	BD
Mouse CD8α		53-6.7	IgG_{2a}	Rat	IHC	BD
Mouse CD8α	APC	53-6.7	IgG_{2a}	Rat	FC	BD
Mouse TCR vα2	APC	B20.1	IgG_{2a}	Rat	FC	Caltag
Mouse TCR vβ5.1, 5.2	PE	MR9-4	IgG_1	Mouse	FC	BD
Mouse TCR vβ8.1, 8.2	PE	MR5-2	IgG_{2a}	Mouse	FC	BD
Myc-tag		9E10	IgG_1	Mouse	*In vivo* depletion	Hybridoma su-pernatant (ATCC CRL-1729)
Myc-tag		3A7	IgG_{2a}	Mouse	*In vitro* depletion	US Biological
Myc-tag			Polyclonal	Rabbit	FC	Santa Cruz
Ovalbumin			Polyclonal	Rabbit	IHC	Acris
Rabbit IgG	PE		Polyclonal	Goat	FC	Santa Cruz
Mouse IgG Fc			Polyclonal	Rabbit	*In vitro* depletion	Jackson Immu-noResearch

2.2 Methods

2.2.1 Molecular biology

Polymerase chain reaction (PCR)

Site-directed mutagenesis PCR using overlapping primers was performed to insert a myc-tag sequence into the TCR or to generate vectors in which the TCRα and TCRβ chains were linked by a P2A element. For this, two separate PCRs (PCR1 and PCR2) were carried out yielding partially overlapping fragments which at the 5' or 3' end contain the newly introduced sequence. In a third reaction (PCR3), the two products were combined in an annealing step resulting in a complete gene carrying the modification, which was then amplified by addition of primers. Figure 4 shows a schematic layout of the procedure.

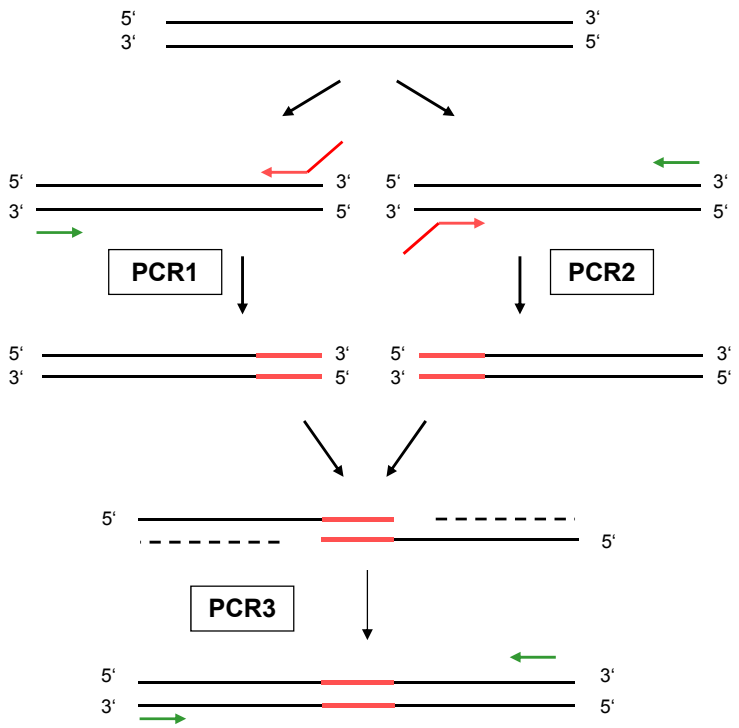

FIGURE 4: Design of site-directed mutagenesis PCR using pairs of overlapping primers. Green: Primers specific for the TCR sequence. Red: Myc-tag or P2A sequence.

Reaction mix PCR1/2:	100	ng	plasmid DNA
	1.5	µl	primer 1 (20 µM)
	1.5	µl	primer 2 (20 µM)
	2	µl	dNTPS (10 mM) (NEBiolabs)
	10	µl	10x Thermo Pol buffer (NEBiolabs)
	2	U	Deep Vent DNA Polymerase (NEBiolabs)
ad	100	µl	H_20

The following primer pairs were used (for sequences see Material section).

Insertion of the myc tag:	PCR1 (fwd / myc1)	PCR2 (rev / myc2)
Cloning of P2A vectors:	PCR1 (fwd / P2Arev)	PCR2 (rev / P2Afwd)

PCR1/2 cycle:	1)	95°C	180 s
	2)	95°C	60 s
	3)	56 - 68°C	60 s
	4)	72°C	60 s / 1 kb product length
	(steps 2 to 4 repeated 30 times)		
	5)	95°C	60 s
	6)	72°C	600 s

DNA products of PCR1 and PCR2 were purified using MinElute PCR Purification Kit (Qiagen) and used for an annealing reaction.

Annealing reaction mix:	100	ng	product PCR1
	100	ng	product PCR2
	2	µl	dNTPS (10 mM) (NEBiolabs)
	10	µl	10x Thermo Pol buffer (NEBiolabs)
	2	U	Deep Vent DNA Polymerase (NEBiolabs)
ad	100	µl	H_20

Annealing cycle:	1)	95°C	180 s
	2)	95°C	60 s
	3)	cool down from 95°C to 45°C with 5°C / 30 s	
	4)	72°C	60 s / 1 kb product length
	(steps 2 to 4 repeated 5 times)		

After the annealing reaction, 1.5 µl primer 1 (fwd, 20 µM) and 1.5 µl primer 2 (rev, 20 µM) were added and PCR3 cycle was performed similar to PCR1/2. The product was purified with MinElute PCR Purification Kit. Correct length of all PCR products was confirmed by agarose gel electrophoresis using DNA1-kb-marker (Sigma) as a control.

Enzymatic restriction and dephosphorylation of DNA

DNA restriction was performed either in analytical scale to confirm correct insert orientation after ligation or in a preparative scale to prepare fragments for cloning. Enzymes were purchased from NEBiolabs and Fermentas; buffers and reaction conditions were applied according to the manufacturer. Digestion was usually performed for 1 h. If different buffer conditions needed to be used for two enzymes in one restriction, DNA was first digested with one enzyme, then precipitated with ethanol and centrifugation, and finally taken up in a buffer appropriate for the second enzyme.

Analytical scale reaction mix:		0.5	µg	plasmid DNA
		2	µl	10x reaction buffer
		5	U	restriction enzyme
		0.2	µl	100x BSA (NEBiolabs) if required
	ad	20	µl	H_20
Preparative scale reaction mix:		6	µg	plasmid DNA
		5	µl	10x reaction buffer
		20	U	restriction enzyme
		0.5	µl	100x BSA (NEBiolabs) if required
	ad	50	µl	H_20

Before subsequent ligation, 5' phosphate residues of vector fragments were enzymatically removed to avoid re-ligation of cohesive vector ends.

Dephosphorylation mix:		50	µl	preparative restriction mix
		5	µl	10x dephosphorylation buffer (Roche)
		2	U	Alkaline Phosphatase (Roche)
	ad	100	µl	H_20

Dephosphorylation was performed at 37°C for 30 min.

DNA extraction from agarose

To isolate specific DNA fragments after enzymatic restriction, the whole reaction mix was loaded onto 0.6 to 2% agarose gels containing 0.5 µg/ml ethidium bromide (Serva) and run at 120 V for 15 to 45 min. DNA bands were visualized with ultraviolet (UV) light and fragments of the right size were cut out of the gel. DNA was extracted from gel slices using Easy-Pure DNA purification kit (Biozym).

Ligation of DNA fragments

DNA concentration and length of digested vector and insert were determined. The fragments were combined in a molar vector : insert ratio of 1:3 using 100 ng vector DNA. Ligation was performed with Rapid Ligation Kit (Roche) according to the manufacturer's instructions. Finally, $CaCl_2$-treated, chemo-competent bacteria were transformed with the ligated product.

Phosphorylation and annealing of oligonucleotides for insertion of a second tag

First, oligonucleotides M1 and M2 were separately phosphorylated using T4 kinase for 1 h at 37°C.

Phosphorylation mix:	10	µl	primer M1 (100 µM)
	10	µl	primer M2 (100 µM)
	20	U	T4 polynucleotide kinase (Fermentas)
	4	µl	10x T4 ligase buffer (Fermentas)
	14	µl	H_20

Phosphorylated oligonucleotides were annealed by incubation at 95°C for 5 min and subsequently decreasing temperature slowly to 4°C.

Transformation of bacteria

$CaCl_2$-treated, chemo-competent *Escherichia coli* (*E. coli*) strains XL-1 blue and SCS110 (*dam*-deficient) (both Invitrogen) were thawed on ice. 40 µl of bacterial suspension were mixed with 1 µg DNA for transformation of plasmid DNA or 10 µl of the ligation reaction

mix for transformation of ligated DNA. The mix was incubated on ice for 20 min. Heat shock was performed by incubating the solution at 37°C for 1 min and afterwards on ice. Subsequently, 1 ml of SOC medium was added and bacteria were cultured at 37°C and 220 rpm in an incubator for 1 h. 100 to 1000 µl of the suspension was plated on LB-agar plates containing 100 µg/ml ampicillin (Roth). As all used plasmids expressed *β*-lactamase under control of a bacterial promoter, ampicillin resistance is conferred to transformed bacteria.

SOC medium:	20	mM	glucose (Roth)
	2	%	tryptone (Roth)
	0.5	%	yeast extract (Roth)
	10	mM	NaCl (Roth)
	2.5	mM	KCl (Roth)
	10	mM	$MgCl_2$ (Roth)
	10	mM	$MgSO_4$ (Roth)
LB medium:	1	%	tryptone
	1	%	NaCl
	0.5	%	yeast extract
LB-agar:	1.5	%	agar (Roth)
	in LB medium		

Plasmid DNA preparation from bacteria and sequencing

Isolation of plasmid DNA from *E. coli* was performed using Spin Plasmid Mini Kit (Invitek) for small scale preparations or DNA Maxi Kit (Qiagen) for large scale preparations; both were applied according to the manufacturer's instructions. Amplification of the correct plasmid was verified by enzymatic restriction. Sequencing of plasmid DNA was performed at MWG Biotech using vector-specific primers.

2.2.2 Cell culture

Cultivation and cryo-preservation of cell lines and primary cells

If not stated otherwise, cells were cultured in exponential growth phase at 37°C, 5% CO_2 and 95% humidity in a HERA cell 240 incubator (Kendro Laboratory Products). Suspension cells were passaged and supplied with fresh medium twice a week. For passaging of adherent cells, medium was removed; cells were washed with PBS and treated with 0.05% trypsin-EDTA in

PBS (GIBCO) for 3 min. Medium was added and cells were seeded in tissue culture flasks at a lower density. For cryo-preservation, medium was removed; cells were taken up in FCS with 10% DMSO (Sigma) and transferred into cryo-tubes (Greiner). For 24 h, cryo-tubes were stored in cryo-containers (Nalgene) at -80°C, after which they were placed in liquid nitrogen. To thaw cells, cryo-tubes were incubated at 37°C. Immediately after thawing cells were taken up in 10 ml cold medium, centrifuged and supplied with pre-warmed medium.

Isolation and stimulation of human PBMCs

Human PBMCs were derived from the blood of healthy donors after informed consent. After donation, 50 ml blood were mixed with 50 ml T cell medium. 50-ml centrifugation tubes (Greiner) were filled with 12.5 ml Ficoll separating solution (Biochrom) on which 25 ml of the medium-diluted blood was layered. The tubes were centrifuged at 650 x g with reduced acceleration and deceleration for 20 min. Lymphocytes accumulated at the interphase from which they were removed with a pipette and transferred into a new tube. Cells were washed twice with 50 ml T cell medium and finally taken up in T cell medium containing PAN-FCS. For stimulation, 24-well non-tissue culture plates (Greiner) were coated with anti-hCD3 antibody and anti-hCD28 antibody (5 µg and 1 µg in 0.5 ml PBS per well, respectively) at 37°C for 2 h. Afterwards wells were incubated with 0.5 ml 2% BSA solution at 37°C for 30 min and washed with PBS. 1 x 10^6 PBLs were seeded in 1 ml medium per well and 100 IU/ml rhIL-2 were added.

Isolation and stimulation of human natural killer (NK) cells

NK cells were isolated from Ficoll gradient-separated PBMCs. For this, 2 x 10^7 PBMCs were taken up in 20 ml T cell medium, seeded in a T150 tissue culture flask (Techno Plastic Products) and incubated at 37°C in a horizontal position for exactly 30 min thus allowing monocytes and dendritic cells to adhere to the cell culture plastic. Subsequently, the non-attached lymphoid cells were carefully removed and counted. Per well of a 6-well plate 1.5 x 10^6 PBLs were seeded in a volume of 5 ml together with 3 x 10^5 interleukin-12-producing RPMI 8866 feeder cells which had been irradiated with 30 Gy. NK cells were cultured for 5 to 6 days and

then stimulated by addition of 100 IU/ml rhIL-2 for another 24 h. This method reproducibly yielded a culture containing 50 to 60% CD3-negative/CD56-positive NK cells.

Isolation and stimulation of murine splenocytes

Mice were sacrificed, spleens removed and placed into a 3-cm dish containing RPMI 1640 medium. Organs were minced and the cell suspension centrifuged at 200 x g for 5 min. Cells were taken up in 2 ml ACK lysis buffer per spleen for 90 s to lyse red blood cells. The reaction was stopped by addition of 25 to 50 ml medium. After centrifugation cells were washed in 20 ml medium, filtered using a cell strainer (BD) and finally seeded in T150 tissue culture flasks at a density of 2 x 10^6 cells/ml. For stimulation, 1 µg/ml anti-mCD3 antibody, 0.1 µg/ml anti-mCD28 antibody and 10 IU/ml rhIL-2 were added.

ACK lysis buffer:

150	mM	NH_4Cl (Merck)
1	mM	$KHCO_3$ (Roth)
0.1	mM	Na_2EDTA (Roth)

pH 7.2

Transient transfection by calcium phosphate precipitation

For transient transfection of cells, calcium phosphate precipitation was used. Per well, 7 to 9 x 10^5 Plat-E or 293T cells were seeded in 3 ml medium into 6-well plates one day before transfection. This way, cells were about 60% confluent at the time point of transfection.

Precipitation mix per well:

	18	µg	total DNA
	15	µl	$CaCl_2$ 2.5M (Sigma)
ad	150	µl	H_2O

After mixing DNA, $CaCl_2$ and H_2O in 15-ml polystyrene tubes (Greiner), 150 µl transfection buffer were added dropwise while vortexing the mix. After 15 min at room temperature, when DNA had complexed with $CaPO_4$ precipitates, 300 µl were added per 6-well. After 6 h incubation, medium was exchanged.

Transfection buffer:	16	g	NaCl
	740	mg	KCl
	500	mg	$NaHCO_3$ (Roth)
	10	g	HEPES
ad	1	l	H_2O
	pH 6.75		

Production of retrovirus supernatant and transduction of T cells

For transduction of murine cells, ecotropic retrovirus was produced by transient transfection of the packaging cell line Plat-E with 18 µg viral vector DNA per well of a 6-well plate. Human T cells were transduced with supernatant generated by transiently transfecting 293T cells with 6 µg pcDNA3.1gag/pol, 6 µg pALF-10A1GaV and 6 µg viral vector DNA per well of a 6-well plate. 48 h after transfection, virus supernatant was harvested, filtered using 0.45-µm pore-size filters (Whatman) and either used directly for transduction or stored at -80°C until use.

To increase transduction efficiency, 6-well or 24-well non-tissue culture plates were coated with 25 µg/ml RetroNectin CH-296 (RN, TaKaRa Biomedicals) by adding 400 µl to 24-wells or 1 ml to 6-wells and incubating for 2 h at room temperature. For blocking the same volume of a 2% BSA solution was added and plates were incubated at 37°C for 30 min. After rinsing the wells with 2.5% HEPES in PBS, cells and viral supernatant were added. To facilitate efficient fusion of the retrovirus envelope with the cell membrane, 4 µg/ml protamine sulphate was applied. Then, plates were spinoculated at 800 x g and 32°C for 90 min.

Depending on the cell type to transduce, different protocols were employed.

i) Cell lines were generally transduced once in RN-coated 24-wells using 1 x 10^5 cells in 1 ml medium and 1 ml viral supernatant per well.

ii) Murine splenocytes were transduced twice at day 1 and day 2 after isolation. For this, 6 x 10^6 cells in 0.5 ml medium were seeded in RN-coated 6-wells and 3 ml virus supernatant was added per well. At the time point of the first transduction, anti-mCD3 and anti-mCD28 antibody and rhIL-2 were added at the same concentration as used for stimulation. For the second transduction, 3 ml medium was re-

moved from the wells and substituted with 3 ml new virus supernatant. This time, only rhIL-2 was used.

iii) The first transduction of human PBLs was performed 48 h after isolation by adding 1 ml virus supernatant supplemented with 100 IU rhIL-2 to the cells cultured in the antibody-coated 24-wells. For the second transduction 24 h later, the cells of one well were split into three and seeded into RN-coated 24-well plates to which 1 ml rhIL-2 containing virus supernatant was added.

Expression of the virus-encoded transgene was usually analyzed 72 h after transduction by flow cytometry.

Flow cytometry

For staining of cell surface antigens, about 5 x 10^5 cells were incubated with 1 µg antibody in 100 µl PBS at 4°C for 20 to 40 min. Subsequently cells were washed twice in 1 ml PBS and, if necessary, the procedure was repeated with a secondary antibody. Finally cells were taken up in 200 µl PBS and fluorescence intensity was measured with a FACSCalibur device and CellQuestPro software (BD). Data analysis was performed with FlowJo software (Tree Star). To discriminate between living and dead cells, staining with propidium iodide (PI, Sigma) or 7-amino-actinomycin D (7-AAD, BD) was accomplished by adding the substances to the cells 10 min before measurement without a washing step.

For the flow cytometric analysis of peripheral lymphocytes of mice, 50 µl blood were mixed with 50 µl PBS and 1 µg antibody and incubated at room temperature for 30 min. If blood was derived from Rag-$1^{-/-}$ mice, samples were pre-incubated with 1 µg anti-CD16/32 antibody for 5 min to block Fc receptors. Red blood cells were lysed using FACS Lyse/Wash Assistant (BD) and samples were measured as described.

Fluorescence-activated cells sorting (FACS)

FACS was performed to enrich cells for a certain surface antigen using a specific antibody. For this, about 2 x 10^7 cells were centrifuged, taken up in 500 µl PBS and about 10 to 20 µg of

antibody were added. Cells were incubated at 4°C for 30 min and subsequently washed twice in 10 ml FACS-PBS. If necessary, the labeling was repeated with a secondary antibody. Before sorting, cells were filtered using a cell strainer, centrifuged and finally taken up in 2 ml FACS-PBS. Sorting was performed with a FACSAria or FACSVantage device (both BD). Sorted cells were collected in FACS-RPMI, centrifuged and taken up in the appropriate medium additionally supplemented with 10 µg/ml gentamycin (Gibco) and 200 U/ml Pen/Strep to prevent bacterial contamination. Sorting results were analyzed by flow cytometry.

FACS-PBS:	1	%	inactivated FCS
	200	U/ml	antibiotic / fungicide mix (Gibco) in PBS

FACS-RPMI:	15	%	inactivated FCS
	200	U/ml	Pen/Strep
	10	µg/ml	gentamycin
	10	mM	HEPES
	in	RPMI + GlutaMAX I	

Magnetic-activated cell sorting (MACS)

MACS was applied for sorting of myc-positive PBLs as they appeared to be too sensitive to FACS. Cells (5 x 10^7 to 1 x 10^9) were centrifuged and taken up in running buffer (0.8 ml per 1 x 10^8 cells) and anti-myc microbeads (200 µl per 1 x 10^8 cells, µMACS c-myc tagged protein isolation kit human, Miltenyi Biotec). After 20 min incubation at 4°C, PBLs were washed twice in running buffer, filtered using a cells strainer and finally taken up in 500 µl running buffer. MACS LS separation columns (Miltenyi Biotech) were fixed in a magnet (Miltenyi Biotec), equilibrated with 3 ml running buffer and cells were applied. The columns were washed 3 times with 3 ml running buffer, before removing them from the magnet. Bead-labeled cells, that had bound to the column were eluted with 5 ml running buffer, centrifuged and taken up in the appropriate medium. After sorting, PBLs had to be restimulated.

Running buffer:	2	%	EDTA (Roth)
	1	%	FCS
	in	Mg^{2+} and Ca^{2+} free PBS (Gibco)	

Peptide-specific restimulation of PBLs

T2 cells were incubated with 10 µM peptide in serum-free medium for 2 h at 37°C, irradiated with 63 Gy and washed twice. Per well of a 24-well plate, 1 x 10^6 PBLs were seeded together 1 x 10^5 peptide-loaded T2 cells in 1.5 ml medium supplemented with 150 IU/ml rIL-2. Cells proliferated for 1 to 2 weeks; then IL-2 concentration was decreased to 10 IU/ml to allow PBLs to enter resting phase. Immunologic assays were performed 2 to 3 days thereafter.

Cytokine release assay

Target cells (T2, T2-K^b or splenocytes) were incubated with different amounts of peptide in serum-free medium at 37°C for 2 h and washed twice. Per well, 1 x 10^5 effector cells were co-cultured with peptide-loaded targets in a 1:1 ratio in 96-well round-bottom plates (Corning Costar, Munich, Germany) at 37°C for 24 h. The supernatant was harvested, frozen and later tested for human IFN-γ or murine IL-2 amount by enzyme-linked immuno-sorbent assay (ELISA; sensitivity 4 or 2 pg/ml, respectively; eBioscience) according to the manufacturer's instructions.

Complement-mediated cytotoxicity (CDC) assay

Exponentially growing cell lines or Ficoll-purified PBLs were seeded in a 96-well plate (Corning Costar) with 1 x 10^5 cells/well in depletion medium (RPMI 1640 medium supplemented with 25 mM HEPES and 0.3% BSA). Cells were labeled with 1 µg myc-specific antibody/well (clone 3A7) at 4°C for 1 h, washed and incubated with rabbit complement (for murine T cell lines: LOW-TOX-M; for PBLs: Rabbit Complement MA, both Cedarlane) diluted 1:6 to 1:12 in depletion medium at 37°C for 2 h. For live and dead cell discrimination, cells were stained with 1 µg PI or 3 µl 7-AAD for 10 min and analyzed by flow cytometry. Cells incubated with either antibody or complement alone served as controls. Percent of specific depletion was calculated as [% cytotoxicity (antibody+complement) – % cytotoxicity (complement alone)] / [100% – % cytotoxicity (complement alone)] x 100.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

TCR-transduced PBLs which had been enriched by MACS and subsequently restimulated were used as targets and autologous NK cells as effectors. Lysis was performed by incubating 5 x 10^3 ^{51}Cr-labeled target cells (100 µCi per sample) with effector cells in E:T ratios from 50:1 to 2:1 in the presence of 1 µg myc-specific antibody (clone 9E10) and 1 µg rabbit anti-mouse IgG_1-Fc polyclonal antibody. After 4 h co-cultivation, 75 µl of supernatant was transferred onto LumaPlates-96 (Perkin Elmer) which were allowed to air-dry. Scintillation was analyzed using a TopCount device (Perkin Elmer). Spontaneous release was measured by incubating target cells alone, maximum release by directly counting labeled cells. Percent specific lysis was calculated as [cpm (experimental) – cpm (spontaneous)] / [cpm (maximal) – cpm (experimental)] x 100.

Adoptive T cell transfer

If not stated otherwise, RIP-mOVA mice were sub-lethally irradiated with 4 Gy one day before adoptive transfer. Age- and sex-matched recipient mice were injected in the tail vein with 2 x 10^7 (RIP-mOVA mice) or 5 x 10^6 (Rag-$1^{-/-}$ mice) TCR-positive splenocytes (as determined by FACS staining) one day after the second transduction. For depletion of adoptively transferred cells, 500 µg myc-specific antibody (clone 9E10) were injected intraperitoneally (i.p.) 2 d (RIP-mOVA) or 13 d (Rag-$1^{-/-}$) after adoptive transfer. Expansion and depletion of cells was monitored by flow cytometry of blood samples. Diabetes development in RIP-mOVA mice was followed by measuring blood glucose levels with Ascensia ELITE SENSOR strips (Bayer, Leverkusen, Germany). Mice with blood glucose levels higher than 14 mM at two consecutive days were considered diabetic.

Immunohistochemical (IHC) staining

The pancreas of sacrificed mice was embedded in Tissue Tek (Sakura Finetek) and frozen in liquid nitrogen. Microsections of the organs were prepared, mounted on microscope slides and fixed with acetone. Slides were pre-incubated subsequently with Protein Block (Immunotech) and PBS/1% BSA/1% donkey serum. Ova antigen was stained with a polyclonal rab-

bit anti-ova antibody (Acris) and secondary donkey anti-rabbit coupled to Alexa594 (Molecular Probes). CD8-positive cells were detected with rat anti-CD8α antibody (BD) and secondary donkey anti-rat antibody coupled to Alexa488 (Molecular Probes). Nuclei were visualized with DAPI. Images were obtained with Axiovert 200 microscope and AxioVision Rel. 4.5 software (both Zeiss).

2.3 Companies

Acris (Hiddenhausen, Germany)

Amersham (Buckinghamshire, UK)

ATCC (Manassas, USA)

BD (Heidelberg, Germany)

Biochrom (Berlin, Germany)

Biosyntan (Berlin, Germany).

Biozym (Hess. Oldendorf, Germany)

Caltag Laboratories (Karlsruhe, Germany)

Cedarlane (Hornby, Canada)

Charles River (Sulzfeld, Germany)

Chiron (Marburg, Germany)

CILAG (Sulzbach, Germany)

eBioscience (San Diego, USA)

Fermentas (St. Leon-Rot, Germany)

Gibco (Karlsruhe, Germany)

Greiner Bio-One (Frickenhausen, Germany)

Immunomics (Fullerton, USA)

Immunotech (Marseille, France)

Invitek (Berlin, Germany)

Invitrogen (Karlsruhe, Germany

Jackson ImmunoResearch (West Grove, USA)

Merck (Darmstadt, Germany)

Miltenyi Biotec (Bergisch Gladbach, Germany)

Molecular Probes (Karlsruhe, Germany)

MP Biomedicals (Eschwege, Germany)

MWG Biotech (Ebersberg, Germany)

Nalgene (Rochester, USA)

NEBiolabs (Frankfurt a.M., Germany)

PAN Biotech (Aidenbach, Germany)

Perkin Elmer (Waltham, USA)

Qiagen (Hilden, Germany)

Roche (Grenzach-Whylen, Germany)

Roth (Karlsruhe, Germany)

Sakura Finetek (Zoeterwoude, Netherlands)

Santa Cruz (Santa Cruz, USA)

Sigma (Taufkirchen, Germany)

TaKaRa Biomedicals (Otsu, Japan)

Techno Plastic Products (Trasadingen, Switzerland)

The Jackson Laboratory (Bar Harbor, USA)

TIB MOLBIOL (Berlin, Germany)

Tree Star (Ashland, USA)

Whatman (Middlesex, UK)

Zeiss (Oberkochen, Germany)

3 Results

3.1 Introduction of a myc-tag into different positions of the murine P14 TCR

In this thesis, the amino acid (aa) sequence 410 - 419 of the human c-myc protein (myc-tag) sought to be introduced into the structure of a TCR in a position where it can be recognized by a myc-specific antibody without interfering with TCR function. For this, crystal structures of human and murine TCRs [3] were inspected visually. Four different regions were identified, which (1) were protruding from the TCR structure and therefore seemed more likely to be accessible for an antibody and (2) were located outside of the CDR regions, which are primarily responsible for the binding to the peptide-MHC complex. Figure 5 depicts these four regions - namely the N-termini of the TCRα- and TCRβ-chain, the FG loop of Cβ and the c-strand of Cα - in the crystal structure of the 2C TCR [6].

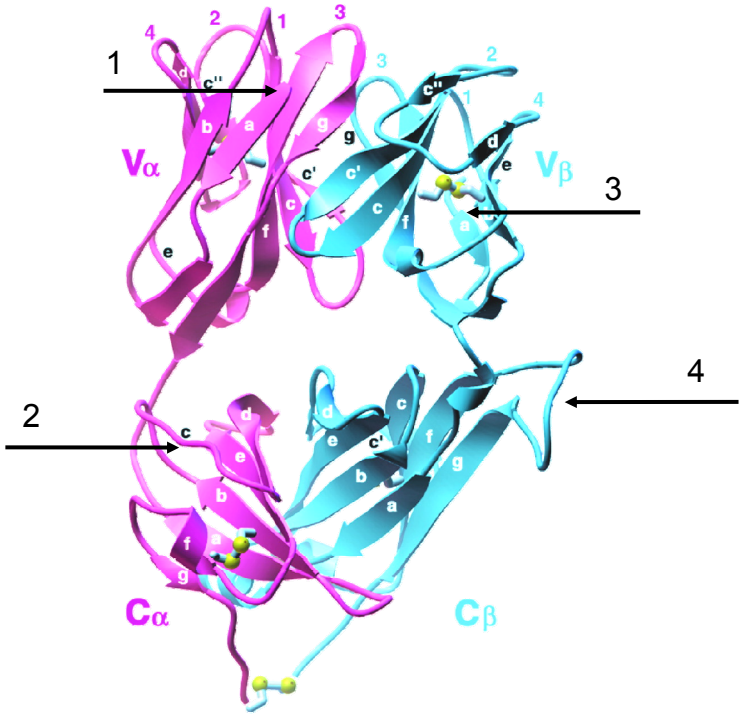

FIGURE 5: Regions for insertion of a myc-tag into a TCR. Crystal structure of the 2C TCRα-chain (purple) and TCRβ-chain (blue). The arrows indicate the four regions selected for insertion of the myc-tag. (1: N-terminus of TCRα-chain, 2: c-strand of Cα, 3: N-terminus of TCRβ-chain, 4: FG loop of Cβ). Adapted from [6].

As a model, the murine P14 TCR (recognizing the glycoprotein-derived peptide 33 - 41 of lymphocytic choriomeningitis virus, gp33) was chosen. For modification of the four described TCR regions two different strategies were applied: either the myc-tag was directly inserted between two amino acids of the TCR sequence, or ten amino acids of the original TCR sequence were replaced by the tag. For some regions, it was tested whether insertion of two adjacent tags was superior to insertion of only one myc-tag regarding the binding of a myc-specific antibody. Finally, nine different myc-tagged TCRs were designed as described in Table 2 and visualized in Figure 6.

TCR chain	Position	Description
P14α	CS	Exchange of aa 170-179 of strand c in the Cα region with one myc-tag
	AN	Fusion of one myc-tag to the N-terminus of the α-chain
	DAN	Fusion of two myc-tags to the N-terminus of the α-chain
P14β	BN	Fusion of one myc-tag to the N-terminus of the β-chain
	L1	Exchange of aa 242-251 of the FG-loop in the Cβ-region with one myc-tag
	L2	Exchange of aa 244-253 of the FG-loop in the Cβ-region with one myc-tag
	L3	Exchange of aa 246-255 of the FG-loop in the Cβ-region with one myc-tag
	DL	Exchange of aa 244-253 of the FG-loop in the Cβ-region with two myc-tags
	XL	Insertion of one myc-tag after aa 248 into the FG-loop of the Cβ-region

TABLE 2: Positions of myc-tag insertion in the murine P14 TCR.

In case of the N-terminal modifications it had to be considered that the first amino acids of the translated protein comprise the signal peptide which is cleaved during TCR processing and export. Therefore, the myc-tag needed to be inserted between the signal peptide and the first amino acid of the mature protein. For identification of the signal peptide cleavage site the SignalP 3.0 software [135] was used. In this program, for each amino acid the probability to be part of a signal peptide (S score), and the probability to be part of a cleavage site (C score) is determined. From these two values the Y score is calculated which estimates where the probability of cleavage is highest. Figure 7A and B show the S, C and Y scores of the P14 TCR chains indicating the putative cleavage position.

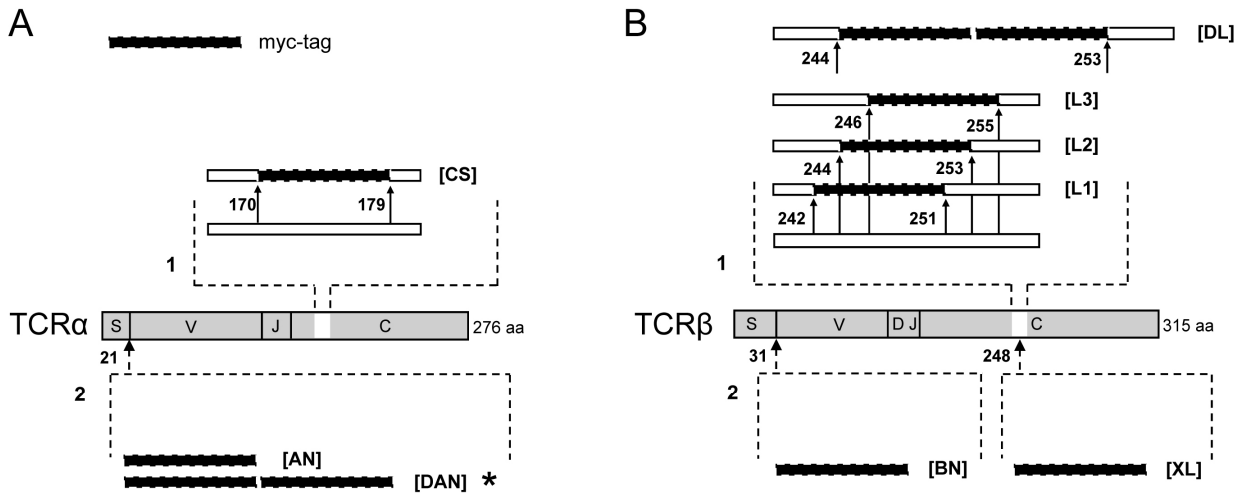

FIGURE 6: Positions of myc-tag insertion in the murine P14 TCR. The 10 aa myc-tag sequence was incorporated into the sequence of the TCRα- (A) or TCRβ-chain (B). In five variants, sequences of the original TCR were exchanged by one or two myc-tag sequences (1). Numbers indicate the first and last aa position of the original sequence that was replaced. Four variants were generated, in which one or two myc-tags were inserted (2). Here, numbers indicate the amino acid position behind which the tag was introduced. The name of each variant according to Table 2 is given in brackets. (S: signal peptide, V: variable region, D: diversity region, J: joining region, C: constant region). The asterix marks the position [DAN] which was later used for modification of different human and murine TCRs.

Furthermore, it was analyzed whether addition of the myc-tag to the N-terminus of the mature proteins leads to changes in the cleavage probability. Therefore, the designed amino acid sequence of the N-terminal myc-tag-modified TCR chains was analyzed using the SignalP 3.0 software. As seen in Figure 7C and D the proposed cleavage site of the signal peptide is not affected by insertion of the myc-tag both in the TCRα- and in the TCRβ-chain.

For retroviral transduction the MP71-PRE vector was employed. This vector harbors the long terminal repeats (LTRs) of mouse myeloproliferative sarcoma virus (MPSV), a leader sequence of murine embryonic stem cell virus and the woodchuck hepatitis virus posttranscriptional regulatory element (PRE) and has been shown to lead to efficient expression of transgenes in murine and human T cell lines and primary lymphocytes [56,61,136,137,138]. Retroviral vectors were generated encoding either the non-modified wild-type (wt) TCR (P14/TCRwt) or the TCR with one of the myc-tag modifications (P14/TCRmyc[X], X being the described position of myc-tag insertion of Table 1). The myc-tag was inserted by site-directed mutagenesis PCR using pairs of overlapping primers. In TCRs with two myc-tags, the second tag was in-

troduced by ligation of the TCR vector containing one myc-tag with a double-stranded oligonucleotide encoding the second tag.

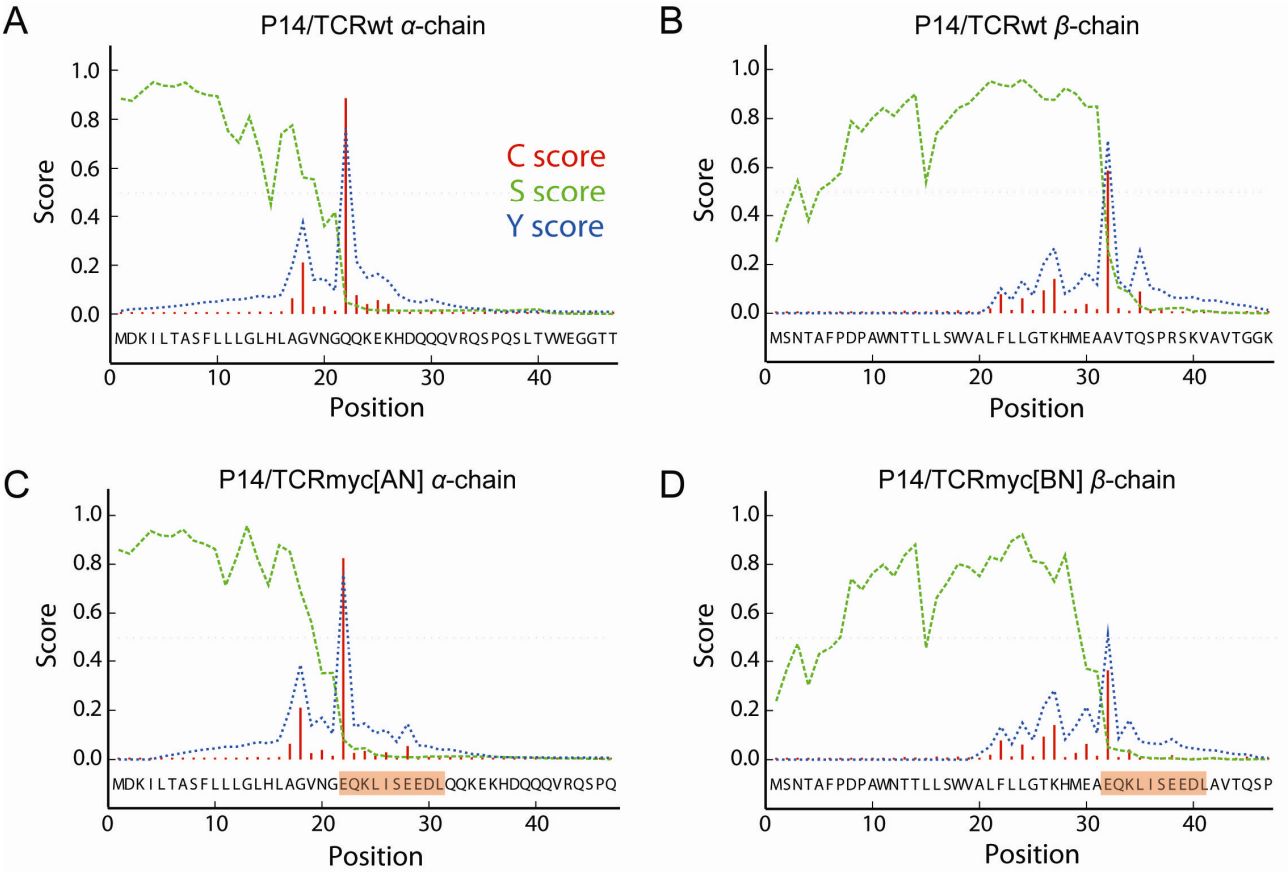

FIGURE 7: Fusion of a myc-tag to the N-terminus of the mature P14 TCRα– and TCRβ–chains does not alter the signal peptide cleavage position. (A,B) The peptide sequence of the P14 TCRα– and TCRβ–chain was analyzed using SignalP 3.0 software. The position with the highest Y score indicates the putative cleavage site of the signal peptide (VNG-QQ for the α–chain and MEA-AV for the β–chain). (C,D) Similarly, the modified P14/TCRmyc[AN] and P14/TCRmyc[BN] amino acid sequences which carry a myc-tag between the proposed signal peptide and the mature protein were analyzed. The myc-tag sequence is boxed in red.

3.2 Expression analysis of myc-tagged P14 TCRs

To analyze whether the myc-tag-modified P14 TCRs can be expressed on T cells, retroviral particles were generated by transfection of Plat-E cells with the TCR gene-containing MP71 vectors, and used to transduce the murine T cell line B3Z. Cells were enriched by FACS sorting using vα2- and vβ8-specific antibodies. Seven days after sorting, expression of the TCR was analyzed by flow cytometry with a vα2- and vβ8-specific antibody.

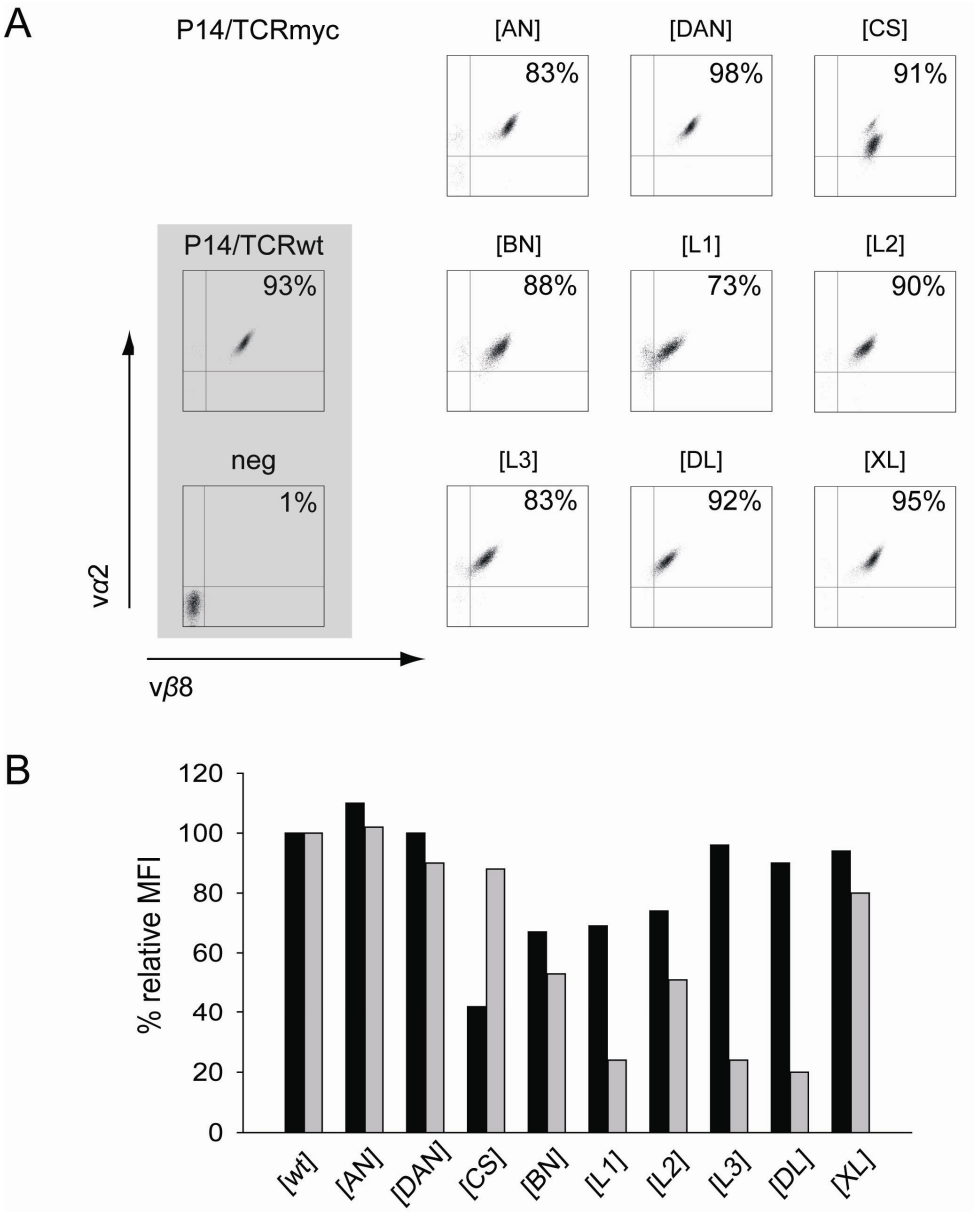

FIGURE 8: All myc-tagged P14 TCRs are expressed in the murine T cell line B3Z. B3Z cells were transduced with one of the P14/TCRmyc and subsequently sorted for vα2 and vβ8 expression. (A) Cells were stained with vα2- and vβ8-specific antibodies and analyzed by flow cytometry. The number of double-positive cells after sorting is given in percentage. Untransduced cells (neg) or cells transduced with P14/TCRwt (both boxed in grey) were used as a control. (B) Mean fluorescence intensity (MFI) of the TCRα-chain (black bars) and TCRβ-chain (grey bars) staining was determined. The depicted relative MFI values indicate the expression level of the myc-tagged TCR relative to expression of the wt TCR chain which was set to 100%.

Figure 8 shows the percentage of TCR-positive T cells as well as the mean fluorescence intensity (MFI) for each TCR chain, which indicates the level of expression. All myc-tagged P14 TCRs were expressed on B3Z cells. Some TCRs (e.g. P14/TCRmyc[AN, DAN and XL] re-

tained a similar expression level of the TCR when compared to P14/TCRwt. However, the MFI of some modified TCR chains (e.g. the β-chain of P14/TCRmyc[DL] or the α-chain of P14/TCRmyc[CS]) was lower when compared to P14/TCRwt.

Next, it was analyzed whether the myc-tag modified TCRs can be detected by a myc-specific antibody. B3Z cells transduced with the different P14/TCRmyc or P14/TCRwt retroviral vectors were stained with a myc-specific antibody and analyzed by flow cytometry (Figure 9).

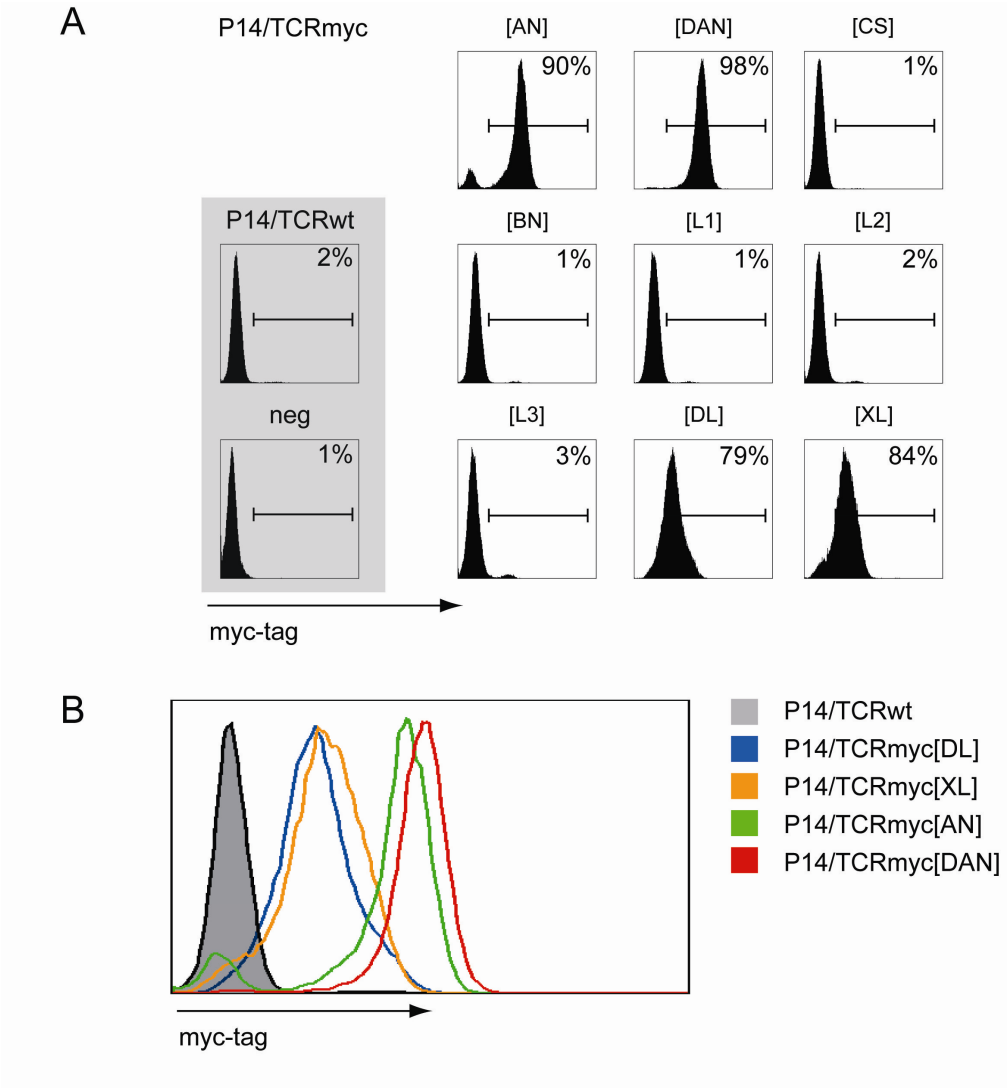

FIGURE 9: Only some myc-tag modified TCRs can be detected by a myc-specific antibody. (A) P14/TCRmyc-transduced and –enriched B3Z cells were analyzed by FACS using a myc-specific antibody. Untransduced cells (neg) or cells transduced with P14/TCRwt (both boxed in grey) were used as controls. Numbers indicate the percentage of sorted cells that stained positive with the antibody. (B) An overlay of the histograms shows distinct levels of myc-tag detection of different TCRs.

Only four of the nine TCRmyc (namely AN, DAN, XL, DL) could be detected with a myc-tag specific antibody.

To reproduce these findings, a second murine T cell line, TCR-deficient 58 cells, was transduced with P14/TCRwt or those myc-tagged P14 TCRs, which bound the myc-specific antibody in B3Z cells (AN, DAN, DL, XL). The cells were sorted by FACS for TCR expression and analyzed as before with vα2- and vβ8-specific antibodies (Figure 10).

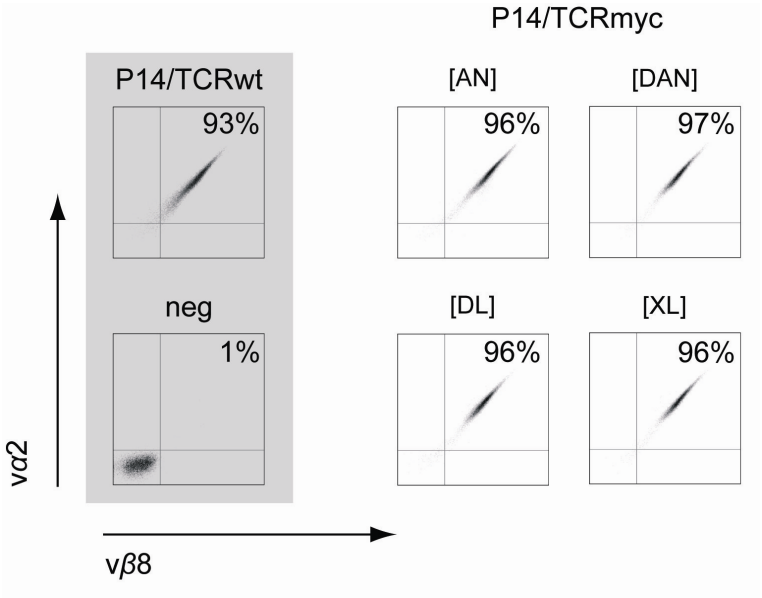

FIGURE 10: P14/TCRmyc [AN, DAN, DL and XL] are expressed in a TCR-deficient T cell line. 58 cells were transduced with P14/TCRmyc [AN, DAN, DL and XL] retroviral vectors and subsequently sorted for vα2 and vβ8 expression. Cells were stained with vα2- and vβ8-specific antibodies and analyzed by flow cytometry. The number of transduced cells after sorting is given in percentage. Untransduced cells (neg) or cells transduced with P14/TCRwt (both boxed in grey) were used as a control.

As in B3Z cells, P14/TCRmyc [AN, DAN, DL and XL] were expressed comparably to the wt TCR. In this experiment, the MFI of the TCR chains did not allow to determine their relative expression level as 58 cells are TCR-deficient. Thus, the introduced TCR does not have to compete with the endogenous TCR for CD3 and TCR export components. Therefore, protein instability due to the introduced modification does not have a similarly strong impact on TCR expression level as observed in B3Z cells, which possess an endogenous TCR [7].

Next, it was analyzed whether the myc-tagged P14 TCRs were also detected by a myc-specific antibody when expressed in 58 cells. Therefore, the transduced cells were stained with a myc-specific antibody and analyzed by flow cytometry (Figure 11).

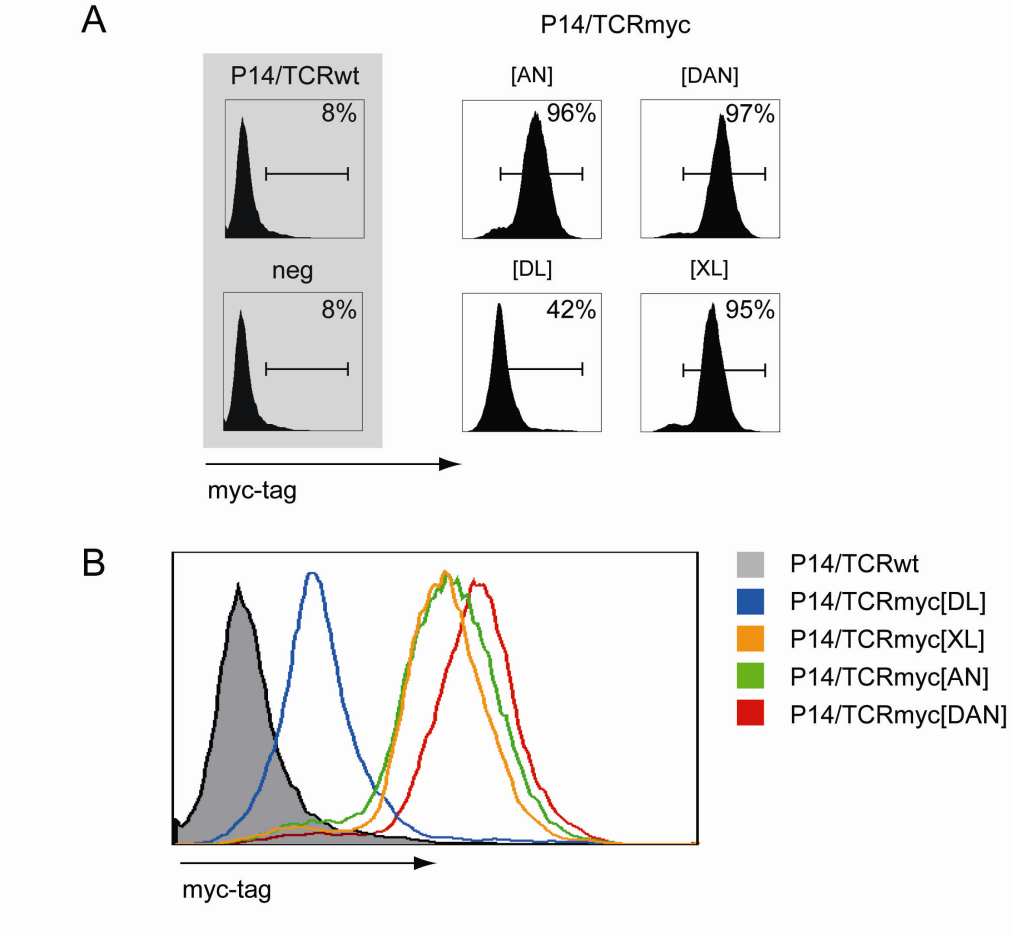

FIGURE 11: P14/TCRmyc [AN, DAN, DL and XL] can be detected by a myc-specific antibody when expressed in a TCR-deficient murine T cell line. (A) P14/TCR-transduced 58 cells that were enriched for TCR expression were analyzed by FACS using a myc-specific antibody. Untransduced cells (neg) or cells transduced with P14/TCRwt (both boxed in grey) were used as controls. Numbers indicate the percentage of sorted cells that stained positive with the antibody. (B) An overlay of the histograms shows distinct levels of myc-tag detection of different TCRs.

Consistent with the previous results, the four myc-tagged P14 TCRs [AN, DAN, DL and XL] could be detected by a myc-specific antibody in the T cell line 58. In both analyzed murine T cell lines the variant [DAN] showed the highest detection level whereas detection of the variant [DL] was lowest. Anti-myc antibody staining of P14/TCRmyc[XL] and P14/TCRmyc[AN] displayed some variation between 58 and B3Z cells. However, in both cell lines the fluorescence intensity of [XL] and [AN] was higher than that of [DL] and lower than that of [DAN].

3.3 Myc-tagged P14 TCRs allow *in vitro* cell depletion

To analyze whether murine T cell lines expressing P14/TCRmyc could be depleted *in vitro*, 58 cells enriched for P14 TCR expression were subjected to complement-mediated lysis. For this, the T cells were incubated first with a myc-specific antibody and subsequently with rabbit complement factors. If antibody has bound to its antigen on the cell surface, complement factors bind to the Fc part of the antibody and induce a signaling cascade leading to specific lysis of the cell. As a control for unspecific lysis, cells were also incubated with complement alone. After incubation, 7-AAD staining was performed to discriminate between viable and dead cells. Flow cytometry results are shown in Figure 12A. Specific lysis was calculated under consideration of unspecific lysis mediated by incubation with complement alone (Figure 12B).

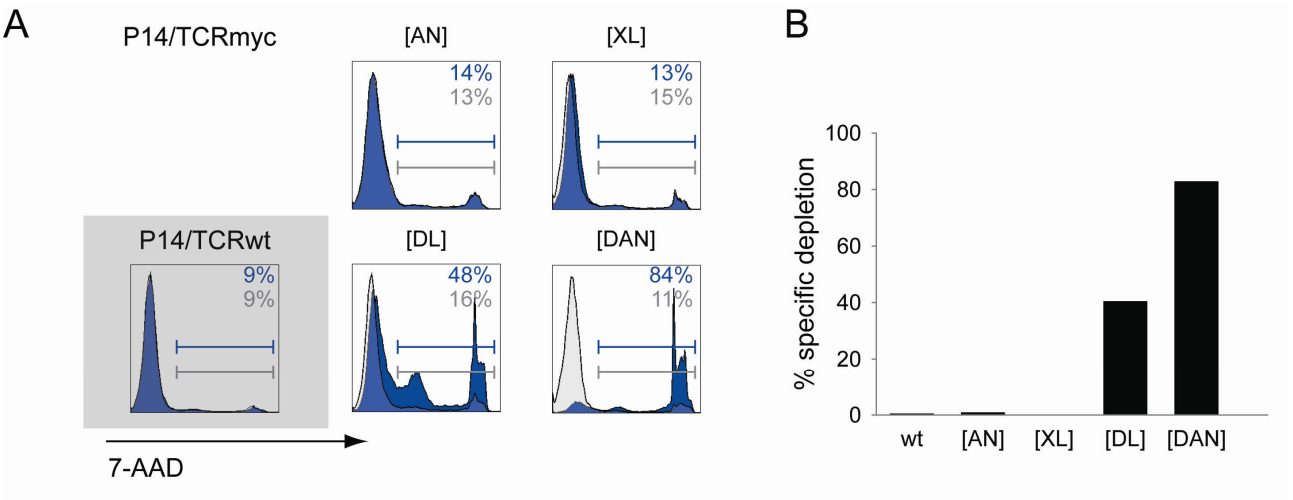

FIGURE 12: P14/TCRmyc[DAN] and [DL] support complement depletion of T cells. (A) 58 cells transduced with P14/TCRmyc [AN, XL, DL or DAN] and enriched for TCR expression were incubated with a myc-specific antibody and rabbit complement factors. Viable and dead cells were discriminated by staining with 7-AAD and FACS analysis. P14/TCRwt-transduced cells were used as a negative control (boxed in grey). Numbers indicate the percentage of dead cells for specific (blue) or unspecific lysis (grey). (B) From these data, percentage of specific depletion was calculated. The experiment was performed twice with reproducible results.

When incubated with complement alone, unspecific cell lysis of 9 to 16% was observed. Specific depletion could only be demonstrated for P14/TCRmyc[DAN] (83%) and [DL] (40%). P14/TCRwt-transduced cells and cells transduced with P14/TCRmyc[AN] and [XL] were not depleted by a myc-specific antibody. Because depletion in the case of P14/TCRmyc[DAN] was most efficient, solely this variant was further studied (now designated as “TCRmyc” only).

3.4 Functionality of myc-tagged P14 TCR is retained

For the potential clinical application of myc-tagged TCRs it is essential that the receptor function is not impaired by the insertion of the tag. For functional characterization of P14/TCRmyc, antigen binding as well as cytokine secretion were analyzed. The first was accomplished by staining with specific peptide-MHC multimers, the latter by detection of IL-2 secretion upon antigen stimulation. For this, the α-chain and β-chain genes of P14/TCRwt and P14/TCRmyc were combined in one single MP71 retroviral vector and linked by the 2A element of porcine teschovirus (P2A) yielding MP71-P14α-P2A-P14β and MP71-P14αmyc-P2A-P14β, respectively. These vectors were used to transduce splenocytes of B6 mice which were subsequently stained with a P14-specific and an irrelevant tetramer. Both TCRs similarly bound the P14 tetramer as shown by comparable MFI in flow cytometry (Figure 13A).

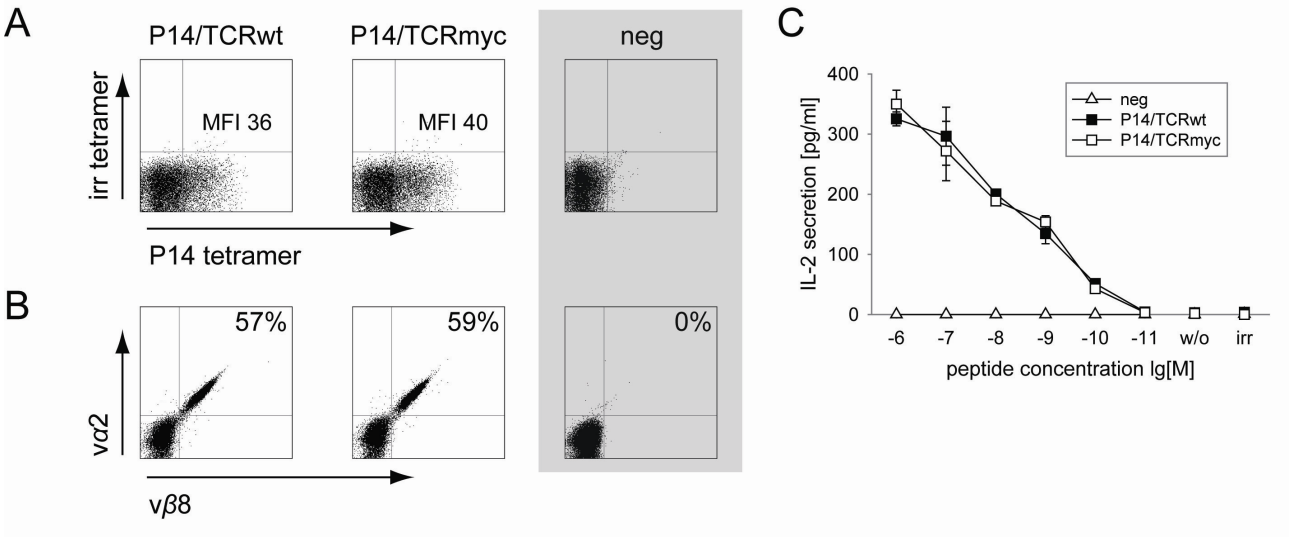

FIGURE 13: P14/TCRmyc functions comparable to P14/TCRwt. (A) B6 splenocytes were transduced with P14/TCRwt or P14/TCRmyc. After 72 hours the cells were stained with a CD8-specific antibody, a P14-specific MHC-tetramer and an irrelevant tetramer (irr). Untransduced cells (neg, boxed in grey) served as a negative control. Cells shown are gated on CD8 expression. Numbers indicate the MFI of the specific tetramer staining. The experiment was repeated twice with similar results. (B) For peptide titration, 58 (CD8α+) cells were transduced with P14/TCRwt or P14/TCRmyc. Cells were stained with vα2- and vβ8-specific antibodies and analyzed by FACS. Transduction efficiency is given in percentage. Untransduced 58 (CD8α+) cells were used as a negative control (neg, boxed in grey). (C) 1 x 10^5 P14/TCRwt- or P14/TCRmyc-transduced or untransduced 58 (CD8α+) cells (neg) were stimulated for 24 hours with 1 x 10^5 B6 splenocytes pulsed with 100 µM to 100 pM gp33 peptide. IL-2 concentration of the culture supernatant was analyzed by ELISA. Unloaded splenocytes cells (w/o) or splenocytes loaded with irrelevant peptide (irr) served as negative controls. Data represent mean values of duplicates and error bars indicate the standard deviation (SD).

For detection of cytokine secretion, CD8α-positive 58 cells were transduced with the P2A-linked P14/TCRmyc or P14/TCRwt retroviral vectors with a similar transduction efficiency of 57-59% (Figure 13B) and stimulated with gp33 peptide-loaded B6 splenocytes. Secretion of murine IL-2 was detected in a peptide concentration-dependent manner and was similar for cells transduced with either TCR (Figure 13C).

3.5 Generation and characterization of OT-I/TCRmyc – a second murine TCR with a myc-tag

To study whether a myc-tag introduced into the same position of a different TCR was also capable of serving as a target site for anti-myc antibody-specific depletion, the murine OT-I TCR (recognizing the ovalbumin-derived peptide 257 – 264, ova) was modified with a myc-tag in position [DAN] as described for the P14 TCR. The OT-I TCR α-chain (wt or myc) and β-chain were cloned into separate MP71 vectors which were used to transduce 58 cells. Cells were enriched for TCR expression with vβ5-specific antibodies by FACS. Flow cytometry analysis using vα2- and vβ5-specific antibodies showed similar expression of both TCRs (Figure 14A). Incubation with a myc-specific antibody revealed only binding to OT-I/TCRmyc-modified cells, but not to OT-I/TCRwt-transduced cells (Figure 14B).

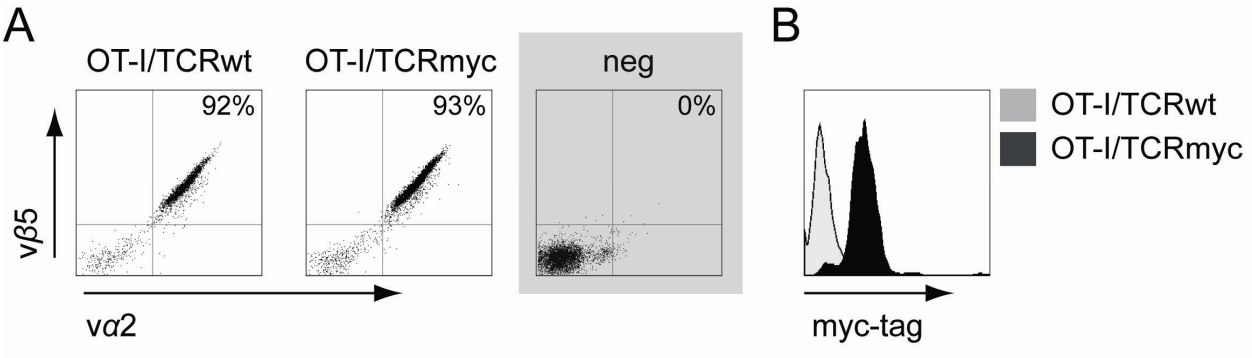

FIGURE 14: Expression of OT-I/TCRmyc and OT-I/TCRwt is comparable. TCR-deficient 58 cells were transduced with OT-I/TCRmyc or OT-I/TCRwt retroviruses and enriched by FACS with a vβ5-specific antibody. TCR expression was detected by staining with vα2- and vβ5-specific antibodies (A) or a myc-specific antibody (B) and anlyzed by flow cytometry. Untransduced 58 cells (neg, boxed in grey) served as a negative control for TCR expression. Numbers indicate the percentage of sorted double-positive T cells.

Furthermore, it was analyzed whether 58 cells transduced with OT-I/TCRmyc can be depleted by a myc-specific antibody *in vitro*. Therefore, the T cells were enriched for OT-I/TCRmyc expression and subjected to complement mediated lysis as described. Depletion was analyzed by 7-AAD staining and flow cytometry (Figure 15A). Specific lysis was calculated under consideration of unspecific lysis mediated by incubation with complement alone (Figure 15B). As seen for P14/TCRmyc, OT-I/TCRmyc-transduced T cells were depleted with a high efficiency by incubation with a myc-specific antibody (78%).

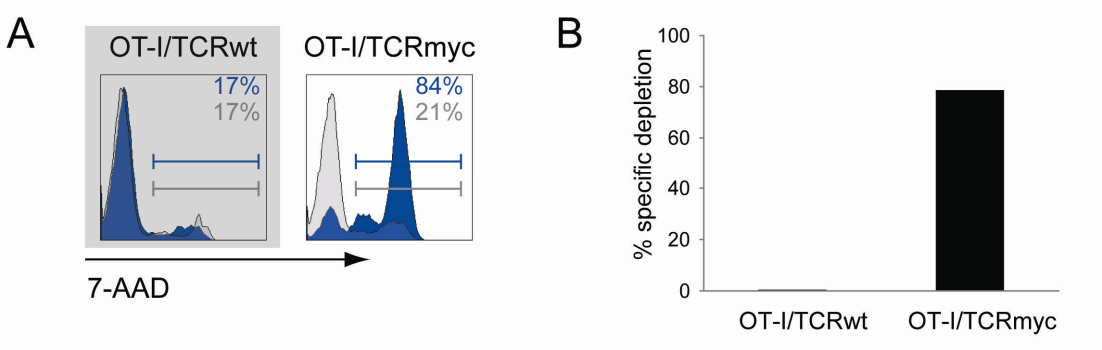

FIGURE 15: OT-I/TCRmyc-transduced 58 cells can be depleted by complement. (A) TCR-deficient 58 cells were transduced with OT-I/TCRmyc and enriched with vβ5-specific antibodies. For depletion, cells were incubated with rabbit complement factors and a myc-specific antibody (blue) or rabbit complement alone as a control (grey). 7-AAD was used to discriminate between living and dead cells. Percentages represent 7-AAD-positive, dead cells. OT-I/TCRwt-transduced cells served as a negative control (boxed in grey). (B) From these data, percentage of specific depletion was calculated. The results represent data from one of at least two independent experiments with comparable results.

To determine whether OT-I/TCRmyc functions comparable to its wt counterpart, specific antigen binding and cytokine secretion upon antigen stimulus were analyzed. For this, B6 splenocytes were transduced with OT-I/TCRmyc or OT-I/TCRwt. 72 hours after transduction cells were stained with an OT-I-specific tetramer and an irrelevant tetramer as a control (Figure 16A).

Cytokine release was analyzed by transducing CD8α-positive 58 cells with the wt or the myc-tagged TCR (Figure 16B), and incubating the cells with ova peptide-loaded T2-K^b cells. After 24 hours IL-2 concentration of the supernatant was determined by ELISA (Figure 16C). OT-I/TCRmyc-transduced cells bound the specific peptide-MHC tetramer comparable to OT-I/TCRwt-transduced cells as indicated by a similar MFI in the FACS staining. Furthermore, IL-2 secretion was similar for both TCRs and was peptide concentration-dependent. Untrans-

duced 58 cells or cells incubated with unloaded T2-K^b cells or an irrelevant peptide did not show significant cytokine release.

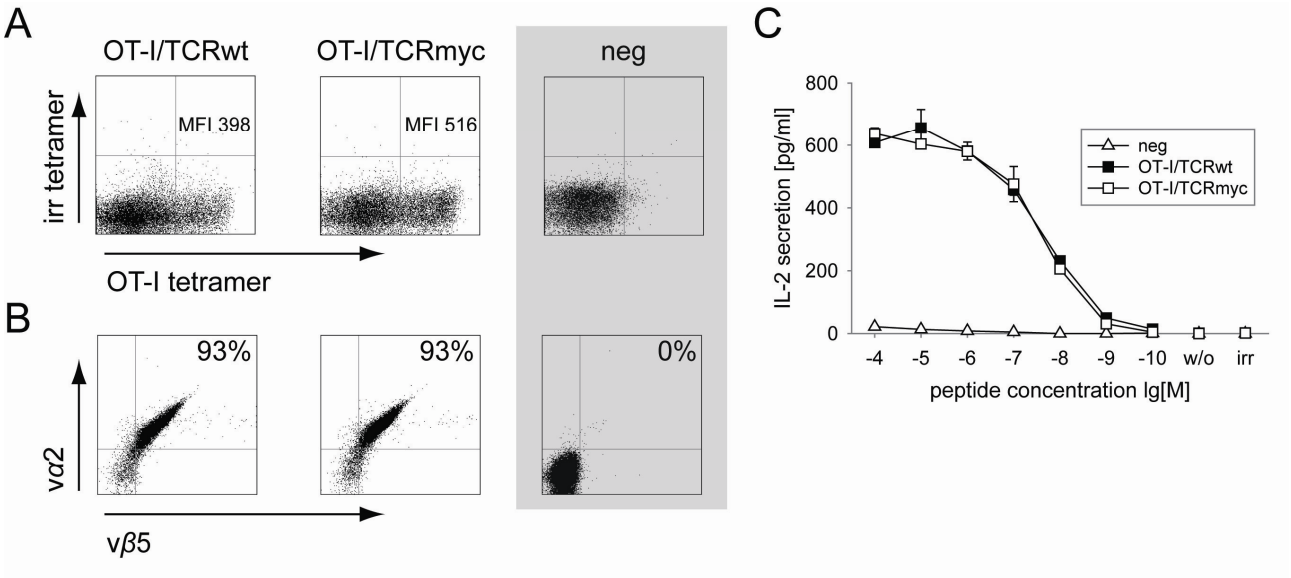

FIGURE 16: Myc-tagged OT-I TCR functions comparable to wt OT-I TCR. (A) B6 splenocytes transduced with OT-I/TCRwt or OT-I/TCRmyc were stained with a CD8-specific antibody, an OT-I-specific tetramer and an irrelevant tetramer. Cells shown are gated on CD8 expression. Untransduced splenocytes (neg) were used as a control (boxed in grey). Numbers indicate the MFI of the tetramer staining. (B) CD8*α*-positive 58 cells were transduced with the myc-tagged or wt OT-I/TCR, stained with v*α*2- and v*β*5-specific antibodies and analyzed by FACS. Numbers show the percentage of transduction efficiency. (C) 1 x 10^5 OT-I/TCRwt- or OT-I/TCRmyc-transduced 58 (CD8*α*+) cells were stimulated for 24 hours with 1 x 10^5 T2-K^b cells pulsed with 100 µM to 100 pM ova peptide. As a control untransduced 58 cells were used (neg). IL-2 concentration of the culture supernatant was analyzed by ELISA. Unloaded T2-K^b cells (w/o) or T2-K^b cells loaded with irrelevant peptide (irr) served as negative target controls. Data represent mean values of duplicates and error bars indicate the SD.

3.6 *In vivo* depletion of T cells transduced with myc-tagged TCRs

Complement-mediated lysis experiments showed that TCRmyc-modified T cells could be depleted by a myc-specific antibody *in vitro*. To analyze whether depletion was also efficient *in vivo*, splenocytes of B6 mice were transduced with either OT-I/TCRwt or OT-I/TCRmyc retroviruses. One day after transduction, cells were injected i.v. into T and B cell-deficient Rag-1$^{-/-}$ mice. This mouse strain was chosen because (i) adoptively transferred T cells can easily be tracked in the blood without the use of congenic markers and (ii) the lymphopenic situation of the mice resembles that of a pre-conditioned patient before adoptive transfer in the clinic.

Blood samples were taken 13 days after injection and stained with a CD8- and a myc-specific antibody (for TCRmyc-transduced T cells) or CD8-, vα2- and vβ5-specific antibodies (for TCRwt-transduced T cells), respectively. Flow cytometry analysis demonstrated the presence of the adoptively transferred cells in the blood of all mice (Figure 17A). For depletion, 500 µg of a myc-specific antibody were injected i.p. and blood samples were analyzed one day later by flow cytometry as described before. In mice that received OT-I/TCRmyc-transduced T cells and antibody, no myc-positive cells could be detected, indicating that TCRmyc-transduced T cells were completely depleted. In contrast, in mice that received OT-I/TCRwt T cells and antibody or in mice, which did not receive antibody treatment, the population of adoptively transferred cells remained unchanged (Figure 17B).

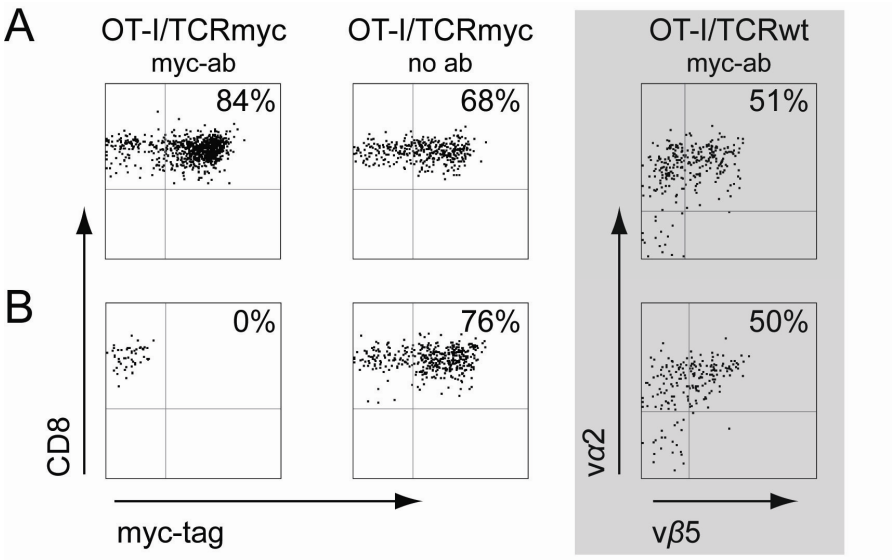

FIGURE 17: T cells transduced with myc-tagged TCRs can be depleted *in vivo*. Splenocytes of B6 mice were transduced with OT-I/TCRmyc. 5 x 10^6 TCR-transduced cells were adoptively transferred i.v. into Rag-$1^{-/-}$ recipients. (A) After 13 days blood was stained for presence of CD8- and myc-positive cells. (B) One group of mice received 500 µg of a myc-specific antibody i.p. for depletion. One day after antibody injection blood samples were collected and analyzed again with CD8- and myc-specific antibodies. As a control, one group of mice received OT-I/TCRwt-transduced cells, which were stained with CD8-, vα2- and vβ5-specific antibodies, and anti-myc antibody treatment (boxed in grey). The stainings show cells gated on CD8 expression and are representative for one of two treated animals.

3.7 Depletion of TCRmyc-transduced T cells in a mouse model of auto-immune disease

In this work, it has been shown that OT-I/TCRmyc-transduced T cells can be depleted by a myc-specific antibody *in vitro* and *in vivo*, and that their function was comparable to that of OT-I/TCRwt-transduced T cells. On that account, the RIP-mOVA mouse model was chosen to analyze whether myc-tagged TCRs can be used to prevent an auto-immune disease.

This mouse model has been extensively studied with regard to antigen-specific auto-immunity [60,130,139]. RIP-mOVA mice express ovalbumin under the control of the rat insulin promoter (RIP) in the β-islet cells of the pancreas [130]. If transgenic OT-I T cells are transferred into these mice, they develop auto-immune diabetes due to destruction of the insulin-producing cells by the T cells. In this model, disease onset is extremely rapid: as early as day two after adoptive transfer insulitis – defined by infiltration of the islets with lymphocytes – can be detected. Blood glucose values increase from normal to highly glycemic (>14 mM) within 24 hours at day four or five after adoptive transfer and mice have to be sacrificed at day six to ten due to severity of symptoms.

In publications, which show diabetes disease in RIP-mOVA mice due to adoptive transfer of T cells, usually OT-I T cells from OT-I transgenic mice were employed. Only de Witte *et al.* described the transfer of OT-I/TCR-transduced T cells, but here additional infection with ova-expressing viruses was needed to stimulate an ova-specific immune response [140].

Hence, it was first analyzed whether polyclonal B6 T cells which were transduced with the OT-I TCR are capable of inducing diabetes in RIP-mOVA mice. In a first attempt, different numbers of OT-I/TCRwt-transduced splenocytes were injected. However, no increase in blood glucose levels could be observed although the same number of transgenic T cells was sufficient for disease induction. Therefore, it was tested whether pre-treatment of the RIP-mOVA mice with cyclophosphamide or total body irradiation gave the transferred cells an advantage due to homeostatic proliferation in the lymphopenic recipient. Splenocytes of B6 mice were transduced with OT-I/TCRwt retroviruses. One day later, TCR expression was analyzed by flow cytometry using vα2- and vβ5-specific antibodies to determine the percentage of transduced T cells. Either 1.5 x 10^6 or 1.5 x 10^7 OT-I TCR-positive cells were injected i.v. into RIP-mOVA mice of which one group had been sub-lethally irradiated with 5 Gy one day before adoptive

transfer and a second had received 50 mg cyclophosphamide per kg body weight two days before adoptive transfer. As a control, one mouse was treated either with irradiation or cyclophosphamide, but did not receive T cells. Expansion of the OT-I-transduced T cells was followed by staining of blood samples seven days after transfer (Figure 18A). Diabetes onset was controlled by measurement of blood glucose concentration (Figure 18B).

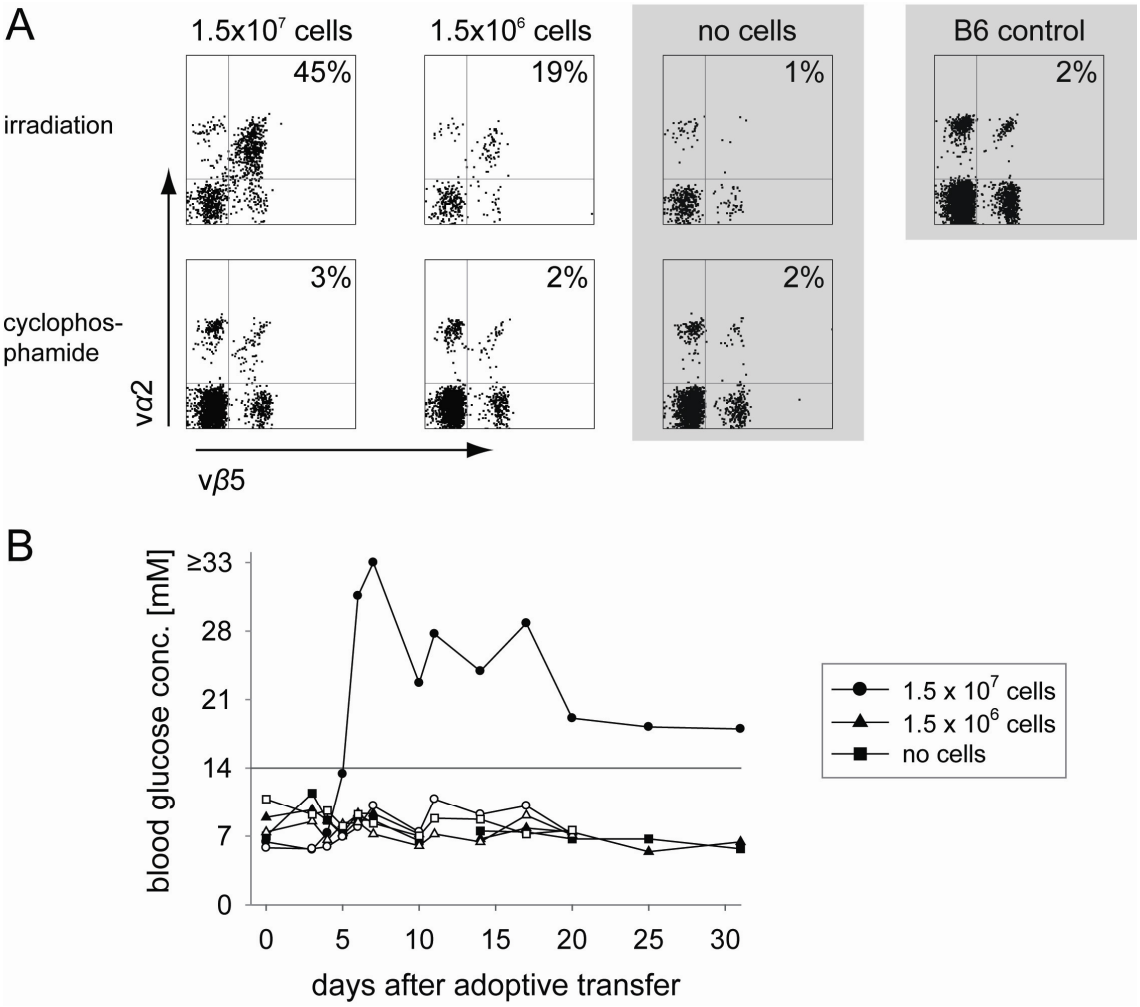

FIGURE 18: OT-I TCR-transduced T cells induce diabetes only in previously irradiated RIP-mOVA mice. B6 splenocytes were transduced with OT-I/TCRwt and 1.5 x 10^7 or 1.5 x 10^6 OT-I TCR-positive T cells were injected i.v. into RIP-mOVA mice (n=1) which had either been irradiated or treated with cyclophosphamide before adoptive transfer. (A) Seven days after transfer blood samples were stained with CD8-, v*α*2- and v*β*5-specific antibodies and analyzed by FACS. As a control blood from mice, which had either been irradiated or received cyclophosphamide but were not injected with T cells, was tested. Blood from an untreated B6 mouse served as a control for the endogenous v*α*2- and v*β*5-positive T cell population. Cells shown are gated on CD8 expression. (B) Blood glucose levels of all mice were determined and followed up to 30 days. The data of irradiated mice are depicted using filled symbols; that of mice treated with cyclophosphamide using open symbols.

Only in mice which had been irradiated, a significant number of vα2/vβ5-positive T cells could be detected in peripheral blood. However, of those mice only the one which had received the higher number of cells showed an increase in blood glucose concentration.

In a second experiment it was analyzed (i) whether it was possible to prevent or cure the T cell-induced diabetes by administration of a myc-specific antibody and (ii) which was the appropriate time-point for antibody treatment. As destruction of islet cells and increase in blood glucose level is very rapid in this model, the time-span between transfer of T cells and administration of the antibody might be crucial. B6 splenocytes were transduced with OT-I/TCRmyc retroviruses. One day later the percentage of vα2/vβ5-positive cells was determined by FACS (data not shown) and 1.5 x 10^7 OT-I TCR-positive T cells were injected into RIP-mOVA mice which had been irradiated using 5 Gy one day earlier. As a control, one mouse did not receive T cells. Blood glucose levels were followed (Figure 19). On day four after T cell transfer, first mice showed increased blood glucose values and were injected i.v. with 500 µg of myc-specific antibody (group "late myc-ab"). One group of mice, which at this time-point still exhibited normal blood glucose concentration, was treated in the same way (group "early myc-ab"). As a control, a third group did not receive antibody treatment. To determine whether multiple administration of antibody was necessary to cure the diabetes, half of the animals in group "late myc-ab" were treated repeatedly (again on days 6, 10 and 13) with 500 µg of myc-specific antibody (group "late myc-ab (rep)").

As seen in Figure 19, only the group of mice which had received the antibody before blood glucose values increased could be effectively treated. After administration, glucose level first increased, but dropped soon and reached normal values at day 30 to 40 after transfer. Mice of this group were further analyzed up to day 100 and no increase in glucose concentration or disease symptoms were observed. Animals which received the antibody at a time-point when they already exhibited high glycemia could not be treated successfully. All mice in the groups "late myc-ab", "late myc-ab (rep)" and "no myc-ab" had to be sacrificed due to severe diabetes symptoms (weakness, loss of weight). These data show, that depletion of T cells via a myc-tagged TCR is able to treat mice suffering from auto-immune T cell-induced diabetes. However, because the chosen model system is so rapid, depletion of the T cells has to be carried out at an early time-point after T cell transfer.

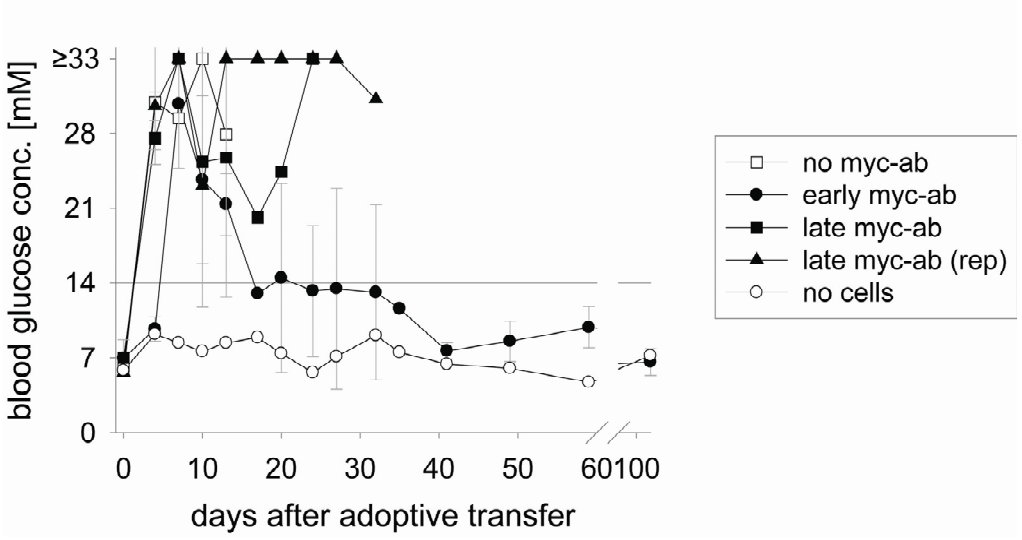

FIGURE 19: Auto-immune diabetes induced by OT-I/TCR-transduced T cells can only be cured by an early administration of antibody. B6 splenocytes were transduced with OT-I/TCRmyc and 1.5 x 10^7 transduced cells were transferred into irradiated RIP-mOVA mice. One mouse did not receive T cells as a negative control. Animals of both groups "late myc-ab" and "early myc-ab" (each n=2) were injected on day 4 after transfer with 500 µg myc-specific antibody. Note that mice in the first one are glycemic at this time-point, whereas in the second one mice show normal blood glucose levels. Mice in group "late myc-ab (rep)" (n=2) received additionally the same dose of antibody on days 6, 10, and 13. As a control animals in group "no myc-ab" (n=2) were not treated with antibody. Blood glucose levels were determined and are depicted as mean values from animals in one group, SD is indicated by the error bars.

Next, the depletion of OT-I/TCRmyc-transduced T cells in RIP-mOVA mice was analyzed in an experiment using larger groups of animals (n=5/group) and compared to OT-I/TCRwt-transduced cells. Furthermore, efficiency of depletion in the pancreas and in lymphoid organs (lymph nodes, spleen) was determined by FACS and IHC staining. For this, splenocytes of B6 mice were transduced with either OT-I/TCRmyc or OT-I/TCRwt retroviruses. One day later, cells were injected i.v. into sub-lethally irradiated RIP-mOVA mice as described. For treatment, 500 µg of a myc-specific antibody were injected i.p. two days after adoptive transfer. None of the animals which received OT-I/TCRmyc T cells and antibody treatment developed diabetes as measured by blood glucose concentration until the end of the observation period on day 100. In contrast, all animals in the control groups receiving either OT-I/TCRwt T cells plus antibody or OT-I/TCRmyc T cells but no antibody succumbed to the disease within four to five days after adoptive T cell transfer and had to be sacrificed two to six days after onset of disease due to severe symptoms (Figure 20).

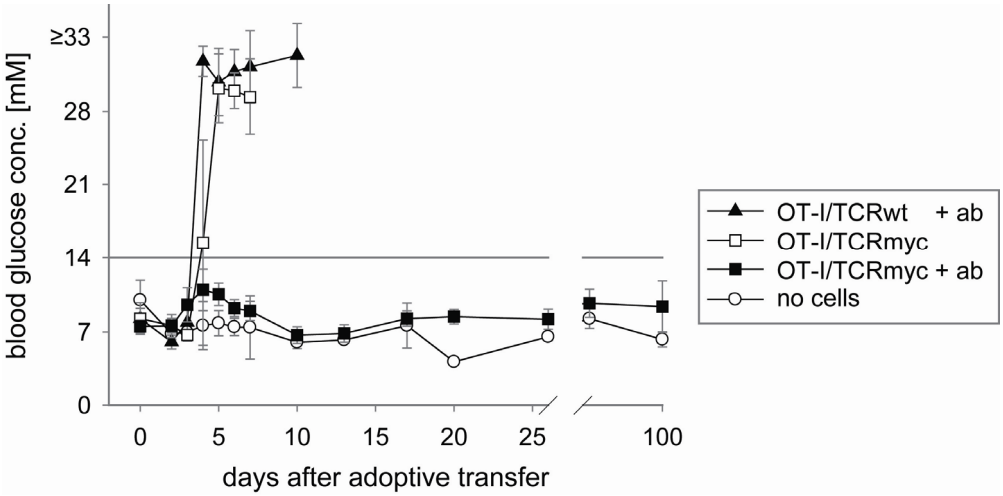

FIGURE 20: Treatment of auto-immune insulitis mediated by myc-specific antibody depletion of OT-I/TCRmyc transduced auto-reactive T cells. B6 splenocytes were transduced with either OT-I/TCRwt or OT-I/TCRmyc retroviruses and 2 x 10^7 TCR-positive cells were injected i.v. into sub-lethally irradiated RIP-mOVA mice. Mice which were irradiated but received no cells served as a negative control. 500 µg of a myc-specific antibody was administered i.p. into all mice that had received T cells harboring the TCRwt and half of the mice which had received T cells carrying the TCRmyc. Blood glucose concentration was determined. Depicted are mean values of all animals (n=5) in one group; error bars indicate SD.

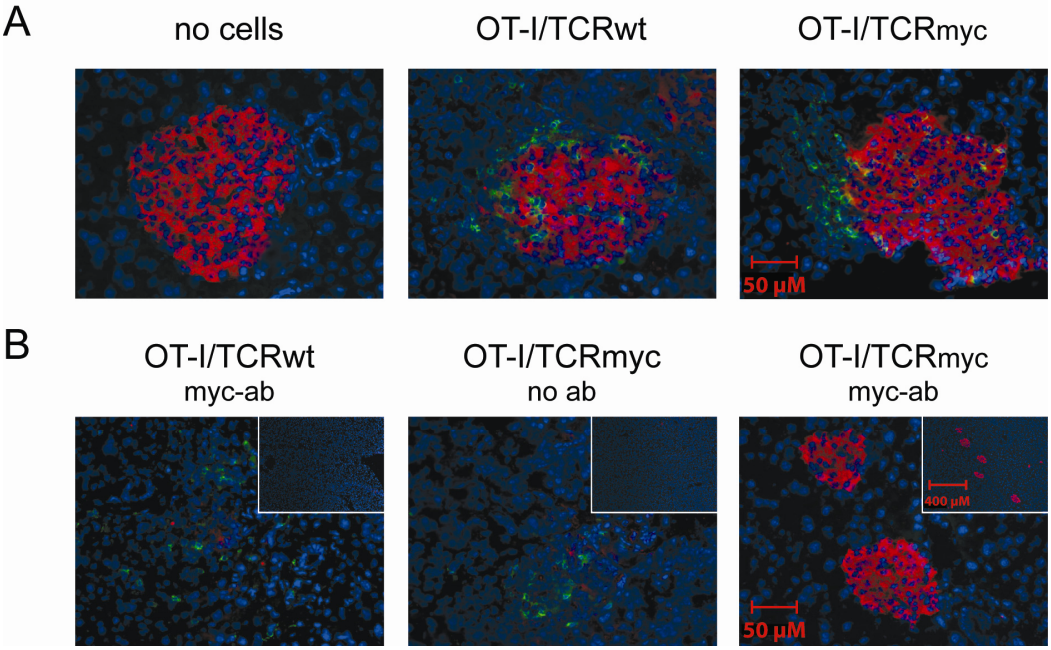

FIGURE 21: Immunohistology shows T cell infiltration and destruction of islets in the pancreas. (A) Two days after adoptive transfer, pancrei of mice from each group were analyzed by IHC with ovalbumin- (red) and CD8- (green) specific antibodies. Nuclei were stained with DAPI (blue). (B) Pancreatic sections from diabetic and antibody-treated mice were stained on day six in the same way. Insets show larger parts of the tissue at a lower magnification.

IHC staining of pancreatic sections of injected mice (but not control animals) two days after transfer showed infiltration of pancreatic islets with CD8-positive T cells demonstrating the very early onset of insulitis (Figure 21A). In stainings from pancrei isolated at day six after transfer from severely sick, diabetic mice (that had either received OT-I/TCRwt-transduced cells plus antibody or OT-I/TCRmyc-transduced cells but no antibody) revealed complete lacking of ova-expressing islet cells due to destruction by OT-I T cells. Also, infiltrating CD8-positive T cells could still be found in the tissue. In contrast, animals that had received TCRmyc-transduced T cells and antibody treatment exhibited intact islet structure and lack of T cells in the pancreas (Figure 21B).

Furthermore, OT-I tetramer-positive T cells were detected in mesenterial lymph nodes (Figure 22A) and spleens (Figure 22B) of diabetic mice, but not in treated animals as shown by flow cytometry.

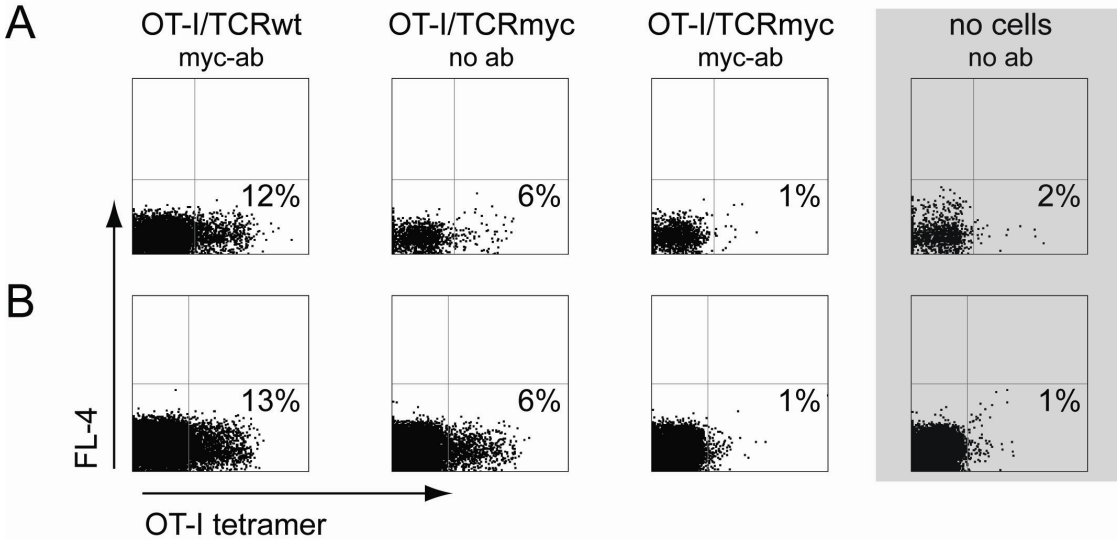

FIGURE 22: Depletion of OT-I/TCRmyc T cells can be detected in mesenterial lymph nodes and spleen. Lymphocytes were isolated from (A) mesenterial lymph nodes and (B) spleen of animals from all groups and analyzed with a CD8-specific antibody and OT-I specific tetramer using flow cytometry. Lymphocytes from mice that did not receive T cells served as a negative control (boxed in grey). Depicted cells are gated on positive CD8 expression.

3.8 Expression and function of the human myc-tagged TCR gp100

So far, murine model TCRs modified with a myc-tag have been analyzed in this study. For the application of the myc-tag safeguard in the clinic, however, it is necessary to determine whether human myc-tagged TCRs retain their function and can be employed for specific cell depletion, as well. For this, a myc-tag was introduced into a human TCR which is reactive against the peptide 209 - 217 of the melanoma antigen gp100. In particular, the gp100 TCR was modified with two myc-tags at the N-terminus of the TCRα-chain – corresponding to the position [DAN] which mediated the depletion of murine T cells transduced with the P14 or OT-I TCR. The gp100 TCRα- and TCRβ–chain genes were cloned into separate MP71 vectors which were used to generate retroviral particles. For expression analysis, the TCR-deficient human T cell line Jurkat76 was transduced. The cells were enriched with a β-chain-specific antibody and sub-cloned by limiting dilution. Several clones were analyzed by flow cytometry using CD3-, myc- and vβ8-specific antibodies. Results of one representative clone are shown in Figure 23. Both, the modified and the wild-type TCR were expressed on Jurkat76 cells as detected with a vβ8-specific antibody (Figure 23A). Because no antibodies are available for the detection of the gp100 TCRα-chain, this staining could not be performed. Only Jurkat76 cells transduced with gp100/TCRmyc, but not cells transduced with gp100/TCRwt could be stained with a myc-specific antibody by flow cytometry (Figure 23B).

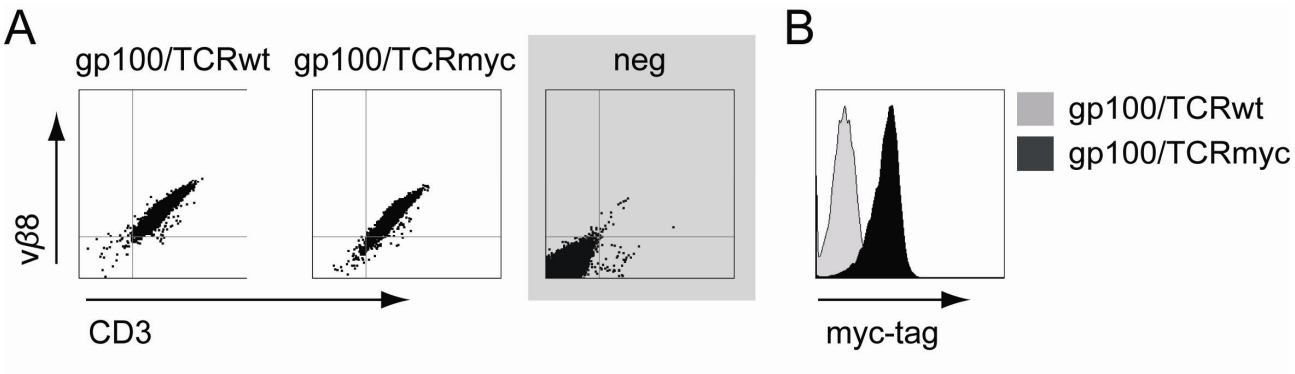

Figure 23: The human gp100/TCRmyc can be expressed comparable to gp100/TCRwt and is detected by a myc-specific antibody. (A) The TCR-deficient human T cell line Jurkat76 was transduced either with gp100/TCRwt or gp100/TCRmyc, enriched with vβ8-chain-specific antibodies and subcloned by limiting dilution. TCR expression was analyzed by flow cytometry staining with a vβ8-specific antibody. Untransduced cells (neg, boxed in grey) served as a negative control. (B) Jurkat76 cells transduced with gp100/TCRmyc were stained with a myc-specific antibody and analyzed by flow cytometry. Cells transduced with the unmodified wild-type receptor served as a control. The data show results of one representative clone of several that were tested.

For functional comparison of gp100/TCRwt and gp100/TCRmyc, human PBLs were transduced with the two TCR retroviral vectors. Antigen binding and cytokine response were analyzed by staining with peptide-MHC tetramers and measuring IFN-γ secretion upon cultivation with peptide-pulsed target cells. Both TCRs similarly bound the gp100 tetramer as demonstrated by comparable MFI in flow cytometry (Figure 24A). Upon stimulation with gp100 peptide-loaded T2 cells, PBLs transduced with either TCR secreted comparable amounts of IFN-γ (Figure 24B).

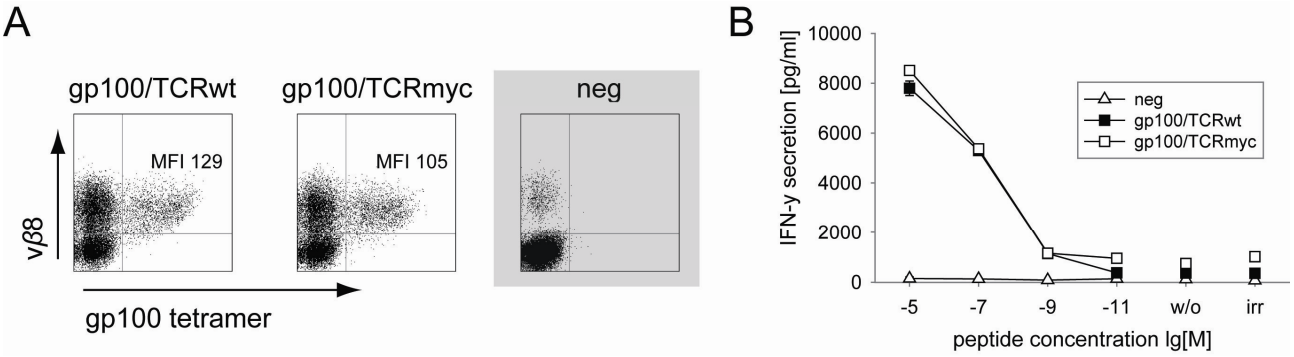

FIGURE 24: The human gp100/TCRmyc functions comparably to its TCRwt counterpart. (A) PBLs were transduced with gp100/TCRwt or gp100/TCRmyc retroviruses and stained with a vβ8-specific antibody and a gp100-specific tetramer. Untransduced PBLs (neg, boxed in grey) show the background of endogenous vβ8-positive T cells. Numbers indicate the MFI of the tetramer staining. (B) gp100/TCRwt- or gp100/TCRmyc-transduced PBLs were co-cultured with T2 cells pulsed with 10 µM to 10 pM gp100 peptide for 24 hours. Untransduced PBLs were used as a negative control (neg). Culture supernatant was analyzed for IFN-γ content by ELISA. Unloaded T2 cells (w/o) or T2 cells loaded with an irrelevant NYeso1-derived peptide (irr) served as negative target controls. Data represent mean values of duplicates and error bars indicate SD. The results were reproduced in two independent experiments and with two different donors.

3.9 *In vitro* depletion of human T cells expressing myc-tagged TCR gp100

To show that gp100/TCRmyc-modified T cells can be depleted by a myc-specific antibody *in vitro*, PBLs were transduced with gp100/TCRmyc retroviruses and enriched using myc-specific MACS beads. Subsequently, the sorted cells were specifically restimulated with gp100 peptide-pulsed T2 cells and IL-2. Seven days after restimulation, PBLs that were 85% to 99%

positive for myc-expression were analyzed for depletion by two different effector mechanisms: CDC and ADCC.

To investigate CDC, a myc-specific antibody and complement factors were added subsequently to the myc-tag-enriched PBLs. 7-AAD staining was performed to discriminate between viable and dead cells and specific lysis was calculated (Figure 25A).

For analysis of ADCC, the T cells were incubated first with a myc-specific antibody and subsequently with NK cells which served as effectors to mediate T cell lysis. To avoid alloreactions, autologous NK cell cultures from the same donor were employed in the assays. To determine specific lysis, the myc-tag-enriched PBLs were radioactively labeled with ^{51}Cr and the release of radioactivity into the supernatant was measured. (Figure 25B). Depending on the assay, 31% to 65% of the gp100/TCRmyc-transduced cells were depleted in the presence of a myc-specific antibody, whereas cells incubated without antibody showed only low unspecific lysis.

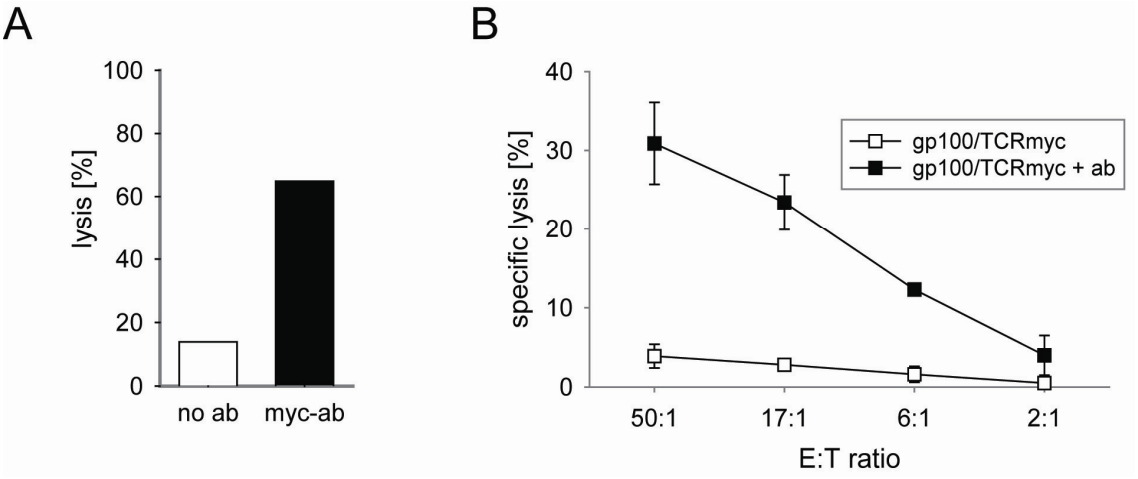

FIGURE 25: T cells transduced with gp100/TCRmyc can be depleted *in vitro* by complement- and cell-mediated lysis. PBLs were transduced with gp100/TCRmyc retroviruses, sorted for myc-positive cells and restimulated with gp100 peptide-pulsed T2 cells. (A) For complement-mediated depletion, cells were incubated with a myc-specific antibody and rabbit complement factors. 7-AAD staining was used to discriminate between living and dead cells. Cells incubated without antibody served as a control. (B) For cell-mediated lysis, autologous PBMCs enriched for NK cells were used as effector cells. ^{51}Chromium-labeled TCRmyc-positive PBLs were incubated with effector cells in E:T ratios from 50:1 to 2:1. A myc-specific antibody and a secondary rabbit anti-mouse IgG1 antibody were added and lysis was measured in a standard four-hour chromium release assay. Samples without antibody served as a control. Data represent mean values of duplicates and error bars indicate the SD. Similar results were obtained in an independent experiment with a different donor.

4 Discussion

Adoptive T cell therapy with TCR-modified T cells is superior to previous cellular immunotherapy approaches for several reasons. First, generation of a large number of gene-modified lymphocytes is rapid and efficient, taking less than two weeks with the current protocols. Second, tumor-reactive T cells can be produced for every patient regardless of pre-existence of TILs. Third, transfer of TCR genes allows endowing T cells with specificities that can be more efficient than those naturally occurring, e.g. high-affine receptors for auto-antigens.

Most likely, results of several clinical trials will be published in the upcoming years. They will not only allow to assess the efficacy of TCR-redirected T cells in humans, but also to define which possible side effects of the therapy are actually relevant in a patient. From one initial clinical study no severe adverse effects were reported [55]. However, the transferred T cells were of low avidity and did not persist long-term in most of the patients [55,141]. Accordingly, a tumor response was detectable, but limited. Progress in the recent years, however, has made it possible to generate TCR-modified T cells with high-affinity TCRs expressed at a high level [52]. Also, culture conditions have been defined to induce long-persistent central memory T cells [138,142]. Increase in efficiency, however, also bears the risk of enhanced side effects, especially auto-immunity. Certainly, with more clinical data available, the need for an inducible termination of therapy will become more obvious. Currently described safety strategies are for many reasons not suitable for TCR-redirected T cells. In this thesis, a novel safety approach was analyzed, based on a TCR-intrinsic mechanism. This safeguard does not impair the function of the T cells and allows their highly specific and efficient elimination *in vivo*.

4.1 Generation and expression of myc-tagged TCRs

Initially, nine different myc-tagged P14 TCR were generated, in which the myc-tag was inserted at several positions in the TCR sequence, either as a single or – to increase anti- myc antibody avidity – as a double tag. The chosen sites were selected (i) to protrude from the compact structure of the variable and constant regions to facilitate antibody accessibility and (ii) not likely to be involved in antigen-recognition which might hamper the function of the modified TCR. When the expression of the myc-tagged TCRs was analyzed in a murine T cell line with antibodies specific for both TCR chains, all could be stained on the cell surface.

However, for some TCRs (e.g. P14/TCRmyc[L1] or P14/TCRmyc[BN]) the detection level was reduced indicating that myc-tag insertion interfered with the stability of the TCR structure leading to a lower expression rate or changed the epitope of the TCR-specific antibody which thus bound with a lower affinity. Staining with a myc-specific antibody was successful in only four of the nine myc-tagged TCRs. In the others, the incorporation into the TCR structure might have forced the myc-tag into a conformation in which it might not be recognized by the antibody. For the myc-specific antibody clone 9E10 it is known that it requires an extended-loop conformation of the epitope to mediate proper binding (personal communication W. Höhne, Charité, Berlin, Germany). Perhaps, the myc-tag adopts this conformation only in some of the TCRs, but not in others. When the tag was fused to the N-terminus of the TCRα-chain, detection with a myc-specific antibody was augmented when two tags were inserted as compared to one. This indicates that employing two adjacent tags might indeed increase antibody avidity to the T cell.

The myc-tagged TCR that was recognized best by the myc-specific antibody and later found to mediate sufficient depletion (P14/TCRmyc[DAN]) showed no decrease in expression level. Similar results were obtained when the murine TCR OT-I and the human TCR gp100 were modified with two myc-tags in the same position.

4.2 Depletion of TCRmyc-transduced T cells *in vitro*

The four myc-tagged P14 TCRs that were detected by a myc-specific antibody were analyzed for their ability to mediate depletion of transduced T cells. Performing a complement depletion assay, significant cell lysis was only achieved by the TCR vectors that carried two myc-tags (P14/TCRmyc[DL] and P14/TCRmyc[DAN]). It is known that the amount of epitopes available for the antibody is crucial for complement-mediated depletion because the cross-linking of several antibody constant regions augments activation of C1q, the initial factor of the classical complement pathway [143]. Most likely, the presence of two tags provides more epitopes in close proximity and may facilitate the parallel binding of two antibody molecules which is beneficial for depletion.

Complement depletion of T cells transduced with P14/TCRmyc[DAN] was of higher efficacy compared to T cells expressing P14/TCRmyc[DL]. Therefore, this position of myc-tag inser-

tion was studied further and used to modify additional TCRs. Transduction of human and murine T cells with gp100/TCRmyc[DAN] and OT-I/TCRmyc[DAN], respectively, also allowed efficient elimination of the lymphocytes by treatment with myc-specific antibody and complement. Compared to the murine T cell lines, the percentage of depletion was lower for human PBLs (about 80% versus 65%). One reason for this is that the purity of myc-positive cells after enrichment was generally higher for the T cell lines and that the remaining myc-negative cells in the culture could not be eliminated. Probably using a second sorting step would overcome this problem. Another point may be the influence of T cell-intrinsic factors, such as activation state, cell cycle phase and expression of anti-apoptotic molecules. In this regard, a T cell line comprises a much more homogenous population of cells than PBLs. Furthermore, the capacity of rabbit complement factors to lyse murine T cells might be higher as compared to human. Still, given the time after which depletion was analyzed (two hours), the overall efficacy of T cell lysis by the TCRmyc safety approach is very high. Assays in which the potential of the HSV-TK gene is analyzed are usually performed for several days to achieve similar results [105,106,144].

To demonstrate that depletion of TCRmyc-transduced T cells can also employ other effector mechanisms, cell-mediated lysis assays were performed with TCRmyc-transduced PBLs as targets and autologous NK cells as effectors. Specific lysis of the PBLs was observed though this was lower as compared to the complement assays. This, however, might be due to the intrinsic property of the antibody to elicit specific effector functions. Ideally, a myc-specific antibody used for depletion of adoptively transferred T cells in a patient should be able to induce several effector mechanisms with a high efficiency (see also chapter 4.6.1).

4.3 Function of TCRmyc-transduced T cells

A prerequisite for any safeguard for adoptive T cell therapy is that is does not interfere with the function of the T cell. Although numerous crystal structures of TCRs or parts thereof have been published, it still remains unclear how exactly signal transduction within the TCR/CD3 complex is managed [3]. In this study, the structure of the TCR was genetically modified. This might on one hand change the mode of antigen binding or, on the other hand, have an impact on the conformation of the whole molecule rendering it incapable of signal transduction. Therefore, the functionality of each modified receptor was analyzed. Antigen recognition was

assessed by staining transduced primary T cells with fluorescently labeled peptide-MHC tetramers. Both, the number of T cells that bound the tetramer and the intensity of binding were not reduced for the myc-tagged TCRs compared to their wt counterparts suggesting that the myc-tag insertion did not interfere with the recognition of the cognate peptide-MHC complex. Signal transduction was analyzed by measuring the amount of secreted cytokines upon antigenic stimulation of TCRmyc-transduced T cells. For the murine TCRs, the T cell line 58 was employed which secretes detectable amounts of IL-2 upon stimulation. Similarly, in the human system, IFN-γ production by gp100/TCRmyc- transduced primary human T cells was assessed. For every analyzed myc-tagged TCR, the quantity of cytokines upon stimulation with different amounts of antigen was comparable to that of the wt receptor indicating that antigen specificity and signal transduction are not affected by the safeguard.

The finding that function of the T cell is maintained in three different investigated receptors suggests that the position chosen for insertion of the myc-tag might be suitable for most TCRs.

4.4 Depletion of TCRmyc-transduced T cells *in vivo*

The efficiency of antibody-mediated depletion may differ *in vitro* and *in vivo*. First, the local concentration of the antibody, complement components or effector cells can vary. Second, permeability of tissue may be an important issue *in vivo*. Third, while *in vitro* only one effector mechanism at a time was analyzed, several may act together in a patient thereby enhancing depletion. Therefore, the possibility to eliminate auto-reactive T cells by the myc-tag safeguard was determined in the RIP-mOVA mouse model. In contrast to RIP-OVA^{low} mice, these animals have a high expression level of ovalbumin in the pancreas. Hence auto-reactivity is not transient and very rapid. This model was chosen to demonstrate the efficiency of the TCRmyc safety approach under drastic conditions.

Here, auto-immune diabetes by transfer of OT-I TCR-transduced T cells was only inducible by prior total body irradiation of the animals. This is in accordance with data from clinical trials or other mouse models showing that rendering the host lymphopenic provides a proliferative advantage for subsequently transferred cells by eliminating regulatory T cells and other cells competing for a limited pool of cytokines [145,146,147]. For treatment, administration of the

myc-specific antibody was required before increase of blood glucose levels were detectable in order to rescue mice from lethal diabetes. If the antibody was injected at a later time-point, prevention of lethality was not possible. In a clinical setting, this pre-emptive treatment, however, is not feasible. Here, T cell therapy would only be terminated in case severe auto-immune side effects become obvious. Still, several facts underline that this necessity is model-specific. Disease onset in RIP-mOVA mice is extremely rapid: as early as day two after adoptive transfer infiltrating lymphocytes were detected in the pancreatic islets (insulitis). As therapeutic anti-myc antibody administration at this time-point was still possible, it can be assumed that the antibody was able to penetrate the pancreatic tissue and eliminate the TCRmyc-modified T cells *in situ*. Without treatment, blood glucose values increased from normal to highly glycemic (>14 mM) within 24 hours at day four or five after adoptive transfer and mice had to be sacrificed at day six to ten due to severity of symptoms. In a patient, careful analysis of several indicators of auto-immunity should be feasible allowing an earlier time-point of treatment. Also, additional administration of immuno-suppressive drugs – an option which was not studied in the RIP-mOVA model – can support antibody-mediated depletion in case of rapidly progressing auto-reactivity.

Nevertheless, it would be desirable to analyze the myc-tag safeguard system in a mouse model in which disease onset is slower and early symptoms of auto-reactivity can easily be followed. Then, therapeutic instead of prophylactic treatment should be feasible.

4.5 Advantages of the TCRmyc safeguard over others

In chapter 1.4 the properties of existing safety approaches have been discussed. All of them comprise several drawbacks rendering them more or less inappropriate for the use in adoptive transfer of TCR-modified T cells emphasizing the need to develop a more suitable strategy.

The most compelling advantage of using myc-tagged TCRs as a safeguard is that this strategy is TCR-intrinsic. The expression of the transgene is directly linked to the suicide mechanism and no additional genes need to be transferred. This avoids purification steps after transduction as adverse effects are only expected by TCR-modified T cells which automatically carry the safety switch. Furthermore, downmodulation of suicide genes, which has been observed for example for HSV-TK [77,103,125,148]. In the case of TCRmyc, downregulation of the

safeguard is coupled to the loss of transgenic TCR expression. As auto-immunity is caused by activation of the introduced TCR chains, their down-regulation most likely also terminates the side effects.

Immunogenicity, which has been shown to lead to unwanted elimination of cells expressing suicide genes, is unlikely to occur for TCRmyc as the molecule consists entirely of human proteins. Immune reactions against the myc-specific antibody can be avoided by producing partially or fully human antibodies.

A further requirement for a safety system is high specificity and low cytotoxicity meaning the absence of side effects on other cells. Although not yet tested in patients, administration of a myc-specific antibody is not expected to cause tissue damage. First, c-myc is not expressed on the cell surface of a normal cell. Second, as a cell cycle regulator and proto-oncogene its expression is tightly regulated and at a relatively low level in normal cells. Still, it has been shown that the shortest peptide sequence needed to give a strong antibody binding signal of the 9E10 myc-specific antibody clone is KLISEEDL [149] and it cannot be excluded that this sequence is part of an MHC-presented peptide.

In sum, many of the obstacles known for other safety systems can be overcome by the use of myc-tagged TCRs. Important issues related to the implementation of the safeguard into a clinical setting and several limitations of the developed approach are discussed in chapters 4.6 and 4.7.

4.6 Implementation of the safeguard into a clinical setting

Protocols for efficient generation of TCR-modified T cells for clinical use have been successfully established [55] and will not be discussed here. This chapter focuses instead on the specific issues related to the applicability of the TCRmyc safety strategy in patients.

4.6.1 Availability of a myc-specific depleting antibody

Three general effector mechanisms of antibodies have been described: (i) blocking of signal transduction of receptor molecules, (ii) depletion by activation of the complement system and (iii) ADCC. Ideally, an antibody used for elimination of auto-reactive T cells *in vivo* should

elicit all of the three responses to achieve a maximum effect. Which of the two last mechanisms is induced, largely depends on the antibody isotype. Human IgG1 and IgG3 antibodies are both capable of recruiting human NK cells by binding to Fc receptors and inducing ADCC as well as initiating a complement cascade. As IgG3 exhibits a shorter half-life, most antibodies in clinical studies are of the IgG1 isoform [150]. When murine antibodies are used in humans, ADCC is best triggered by the isotype IgG3, and complement lysis by IgG2a [151,152]). IgM antibodies, which are most efficient in complement activation, are not of clinical relevance due to their short serum half life. A disadvantage of the administration of murine antibodies is their immunogenicity. Frequently, the induction of human anti-mouse antibody (HAMA) responses has been observed which may impede subsequent treatment with the same antibody [153]. Hence, attempts have been made to either humanize the antibodies by replacing murine with human sequences, or to obtain fully human antibodies from phage display or human Ig locus-transgenic mice [154].

An alternative option is to make use of an antibody which has already been tested for safety in a clinical trial. In 2005, 18 different monoclonal antibodies had been approved for clinical use in the US and Europe, and more than 150 were in clinical trials [155]. The majority of them targets overexpressed oncoproteins (tumor therapy), cell surface molecules of immune cells (immuno-modulation) or viral proteins (treatment of infections). However, most of these antibodies will not be suitable for the safety strategy described in this work. First, the epitopes of many antibodies have not yet been defined. This, however, is necessary, as only a short target sequence can be introduced into a TCR. Second, high specificity of the antibody for the adoptively transferred T cells is desired. Most likely, antibodies that recognize oncoproteins (e.g. Her2, EGFR) or molecules of the immune system (e.g. CD4, CTLA-4) will provoke unwanted side effects on other cell types. Third, the epitope tag itself should not be immunogenic which can not be excluded if parts of viral proteins are employed. Nevertheless, if one finds an appropriate alternative antibody-tag-combination, their applicability to the TCRmyc safeguard system needs to be analyzed.

4.6.2 Universality of the safeguard for different TCRs

Ideally, the TCRmyc safety approach should be applicable to every TCR employed in clinical studies. In this study, the myc-tag has been fused to one TCR chain N-terminus which is part

of the variable region. Every TCR used for therapy, however, has its unique variable domain; and although all are expected to adopt an Ig-like fold, minor differences in the tertiary structure exist and not all TCR variable segments have been crystallized. It is not clear whether the insertion of a myc-tag will maintain expression and function of every therapeutic TCR and will allow efficient depletion of transduced T cells. In this thesis, two mouse TCRs with two different TCRα-chains belonging to the same variable region familiy (vα2) and one human TCR have been analyzed; and all tested TCRs were suitable for application of the safeguard. Still, it would be interesting to test TCRs with various TCRα variants to see if differences exist. Probably, for some TCRs the insertion of only one tag might be sufficient for elimination or even required for maintenance of function. If the approach is found not to be universal for all TCRs, it might be necessary to re-evaluate the TCRmyc strategy for every therapeutic receptor going into a clinical trial anew. Instead of introducing the myc-tag into the variable region of a TCR, one might also consider the modification of the constant part to achieve universality. Unfortunately, in this work none of the constant region mutations led to sufficient recognition of the tag by a myc-specific antibody that allowed depletion.

4.7 Eventual limitations of myc-tagged TCRs as a safeguard

4.7.1 Immunogenicity of TCRmyc

A disadvantage with many safety approaches is their immunogenicity as they are usually of viral or bacterial origin or comprise artificial fusion proteins [99,101,102]. An immune reaction against the adoptively transferred cells might lead to their unwanted premature elimination and also prevent the survival of a second graft. Myc-tagged TCRs consist entirely of human protein sequences: the rearranged $\alpha\beta$ TCR chains and two 10 aa stretches of the c-myc protein. Therefore, the risk of immunogenicity is rather low. Still, it can not be excluded that at the fusion site between the tandem tags or between the tag and the N-terminus of the TCRα chain an immunogenic peptide in context with some MHC alleles is generated which is recognized by the immune system of the patient. Furthermore, c-myc is a nuclear protein, and central humoral tolerance to nuclear proteins is not that strict [156,157]. Berger *et al.* injected T cells modified with a Fas-FKBP suicide construct into macaques and observed an immune response against the transferred cells. Detailed analysis, however, revealed that this was directed against epitopes that differed between the human and macaque sequence, but not

against the fusion sites [109]. Such an approach could also be used to test the immunogenicity of myc-tagged TCRs. Still, this would not give conclusive data about the reaction of a large number of patients with a multitude of different MHCs. In this work, murine T cells that were modified with murine TCRs fused to the human myc-tag were injected into mice. The sequence of the human myc-tag differs from the corresponding mouse sequence in 3 of 10 aa. Hence, there is the possibility that the transgene is recognized by the mouse immune system as foreign. In the performed experiments, however, the recipient mice were always immunodeficient at the time-point of T cell transfer, either because of the genetic background of the employed mouse strain ($Rag1^{-/-}$ mice) or because of previous treatment with cyclophosphamide or irradiation. Thus, these experiments do not allow assessing the immunogenicity of myc-tagged TCRs.

Nevertheless, it was experienced in clinical trials that adoptively transferred T cells engraft better in lymphopenic patients [41,158] and pre-treatment with non-myeloablative, lymphodepleting drugs before transfer seems to become a standard procedure. In lymphocyte-depleted patients the immunogenicity of any transgene might not be of importance, though. And even repopulating endogenous lymphocytes are likely to be ignorant of foreign peptides due to peripheral tolerance mechanisms.

4.7.2 Elimination of activated T cells

Upon activation of a T cell, the TCR/CD3 complex becomes downmodulated which has been shown to be caused by increased intracellular degradation of the constantly recycling molecules [159]. Thus, efficient elimination of TCRmyc-transduced T cells by a myc-specific antibody might be hampered if the cells are activated due to auto-reactivity and the number of TCRmyc molecules on the surface is reduced. It has been demonstrated that the amount of epitopes is critical for mediating depletion [143]; and also the results presented in this work show that efficient complement lysis was only achieved with the TCRmyc variant that showed highest anti-myc antibody staining suggesting that the quantity of myc-tag epitopes is of importance. In the *in vitro* data presented in this study, however, TCR downmodulation could not be analyzed. The T cells subjected to complement lysis assays were either a T cell line (58 cells) or primary human PBLs. The 58 T cell line barely shows receptor downmodulation upon stimulation (data not shown) and the PBLs were in resting phase, which employs culture

conditions with low IL-2 concentration, at the time-point of the depletion assay. Accordingly, the *in vitro* data do not allow drawing conclusions about the efficiency of elimination of activated TCRmyc T cells. In contrast, it can be supposed that the T cells depleted in the *in vivo* experiments were – at least to some extend – activated. First, the T cells were injected after three days of mitogenic stimulation with anti-CD3/CD28 antibodies and high-dose IL-2. Second, when injected into Rag-1$^{-/-}$ mice, the T cells underwent homeostatic lymphopenia-induced expansion which correlates with an activated phenotype [160]. Third, in RIP-mOVA mice the depletion was carried out at a time-point when the transferred T cells had already migrated to the pancreas and most likely encountered their antigen. Probably, several effector mechanisms are involved in antibody-induced depletion *in vivo* which allows the elimination of activated T cells. Still, it might be interesting to compare the *in vitro* depletion efficacy of activated and resting T cells side by side and to characterize the phenotype of *in vivo* depleted T cells by staining for activation markers.

4.7.3 Elimination of transformed T cells

Retroviral insertion into the genome of a host cell bears the risk of malignant transformation through activation of oncogenes. In this case, it is desirable to have the possibility to eliminate the leukemic T cells in the patient by a safeguard mechanism. In this work, it has not been analyzed whether the TCRmyc safety approach is applicable to treat integration-induced leukemia. The only clinical trial for adoptive cell therapy, in which transformation was observed, is the genetic modification of stem cells from X-SCID patients with the gene for the cytokine receptor common gamma (γc) chain [78,79]. However, several aspects argue against insertional mutagenesis by TCR gene transfer. First, in contrast to the X-SCID studies, the transduced T cells are not hematopoietic progenitors but differentiated lymphocytes. Recent data indicate that transfer of various single oncogenes is detrimental in stem cells, but does not lead to transformation of T cells (personal communication D. v. Laer, Georg-Speyer-Haus, Frankfurt a.M., Germany). Second, it is not yet known whether the TCR transgene does provide a direct proliferative advantage for the T cells whereas expression of the γc chain in stem cells is clearly essential for stimulation by many growth-promoting cytokines [161].

Still, it is not clear whether transformed T lymphocytes might escape myc-specific antibody depletion by either downregulation or loss of TCRmyc expression or acquisition of comple-

ment resistance. Antigen-loss variants have been described in antibody-treated B cell lymphomas [162]; and resistance to antibody therapy has been observed even when the antigen is still expressed [114]. In a transformed T cell, expression of the transgenic TCR might not be essential for T cell survival. Hence, loss of TCRmyc expression might not lead to reduced proliferation but abrogates the possibility of elimination. Some tumor cell lines have been demonstrated to overexpress complement inhibitory molecules rendering them insensitive to complement-mediated lysis [163,164,165]. Further mechanisms of resistance include an elevated apoptotic threshold or altered susceptibility to cellular cytotoxicity.

Thus, it remains desirable to determine the influence of retroviral transfer of TCR genes into human T cells; e.g. by analysis of integration loci, loss or maintenance of polyclonality and growth behavior over an extended period of time. Another possibility is to transfer an oncogene additionally to the TCR genes – as to mimic activation of endogenous oncogenes – and to study whether the T cells become transformed. Then, anti-myc antibody-mediated depletion of these T cells could be analyzed.

4.7.4 Elimination of T cells expressing TCR heterodimers

If a T cell is genetically modified with a therapeutic TCR four different receptor combinations can be expressed: the transgenic TCR, the endogenous TCR and mispaired heterodimers of the α- and β-chain of the transgenic and endogenous TCR. Depending on the "strength" of the TCRs – meaning its intra- and inter-chain stability and interaction with CD3 subunits – only one, several or all of the combinations are found on the T cell surface [61,62]. The safeguard proposed in this thesis work relies on expression of the myc-tagged transgenic TCRα-chain. In an unfortunate scenario, a heterodimer of the endogenous α-chain and the introduced β-chain recognizes an auto-antigen and causes auto-immunity. If this heterodimeric TCR is dominant over the other variants, the myc-tagged TCRα-chain might not be expressed at all, hence providing no possibility of T cell elimination. To exclude this, one might also introduce a tag into the TCRβ-chain. In this study, however, modification of the P14 TCRβ-chain with one myc-tag did not lead to recognition by a myc-specific antibody. Therefore, it needs to be analyzed whether a double myc-tag (as in the P14 TCRα-chain), a flexible linker between the tag and the N-terminus of the TCR chain or the choice of a different tag support the depletion of auto-reactive T cells via the TCRβ-chain.

4.7.5 Activation of auto-reactive T cells by the myc-specific antibody

Several monoclonal antibodies specific for the TCR/CD3 complex have been identified that have both depleting and activating capacity. One example is the anti-human CD3 antibody OKT3 which stimulates human PBLs *in vitro*, but can be used for depletion of CD3-positive T cells in the presence of complement [166,167]. Similarly, the anti-murine CD3ε antibody 145-2C11 acts as a mitogen in T cells *in vitro* and *in vivo*, but is also described to induce immunosuppression by lymphocyte depletion [168,169]. Some factors that determine the effects of an anti-TCR/CD3 antibody are (i) the antibody isotype, (ii) the presence of serum complement factors or cells capable of mediating ADCC and (iii) the dose of the administered antibody [170].

Preliminary experiments revealed that an immobilized myc-specific antibody can – in the absence of complement – induce activation of a TCRmyc-transduced indicator cell line *in vitro* (data not shown). Hence, there is the theoretical risk that administration of a myc-specific antibody might further stimulate auto-reactive TCRmyc T cells *in vivo*. In the performed animal experiments, however, no activation of the transferred T cells was observed. Instead, application of the antibody led to a rapid depletion of the T cells (< 1 day) and prevented onset of auto-immune disease. It might be interesting though to evaluate the activating capacity of a myc-specific antibody on TCRmyc T cells, e.g. by injection of a low dose of antibody, a non-depleting antibody or the use of antibody fragments which lack the parts necessary for depletion. In the absence of auto-reactivity, this would provide a tool to specifically stimulate adoptively transferred tumor-reactive T cells in a patient thereby enhancing an anti-tumor immune response.

4.8 Future prospect

Certainly, several clinical trials using TCR-modified T cells will be carried out in the upcoming years. When efficiency of this therapy improves, an increase in the risk of auto-immune side effects is expected emphasizing the need for a reliable safety strategy. This study demonstrated that the introduction of a short peptide sequence into a TCR molecule allows the specific elimination of T cells that express the TCR while maintaining full function. However, some limitations of this approach have been discussed.

For translation into a clinical setting it will be most important to obtain a clinically approved antibody. Currently, only murine myc-specific antibodies of IgG1 or IgG2a isotype are commercially available. Of those, only the hybridoma of clone 9E10 (an IgG1 antibody) can be obtained from ATCC and the variable fragments of this antibody have been cloned into bacterial expression vectors [171]. However, application of a myc-specific antibody in a clinical trial bears several economic hurdles. First, clinical scale production of the antibody under good manufacturing practice (GMP) conditions will be very cost-intensive. Second, before administration in a clinical trial with TCR-modified T cells it might be necessary to test the general safety of the antibody in humans in a separate study. Third, humanization of the antibody or production of a fully human antibody requires further expenses. With our available means, generation of a murine, humanized or fully human GMP-grade myc-specific antibody is difficult. Therefore, emphasis will be laid on finding an alternative peptide with properties comparable to those of the myc-tag that is recognized by a clinically characterized antibody. If a suitable sequence is found, a model TCR will be modified with the alternative tag and analyzed for maintenance of function and efficiency of elimination. Another important point will be to show the universality of the approach. It would be very interesting to compare myc-tag modified TCRs with different Vα segments. Plenty of TCRs have been isolated from various labs and it should be possible to obtain model TCRs of the most common segments. Also, candidate TCRs that are suggested for clinical trials will be analyzed.

The application of the myc-tag strategy is not limited to TCRs. In fact, several gene therapy approaches using cell surface-expressed transgenes could benefit from a specific safety modality. In the laboratory of H. Abken (Division Tumorgenetics and Immunology, Uniklinik Köln, Cologne, Germany) CAR molecules are currently being modified with a myc-tag and analyzed for their capability to mediate depletion of T cells. Similar to TCRs, CAR-transduced T cells can be employed for adoptive cell therapy, but bear the risk of auto-immune side effects. Additionally, in our laboratory Nicole Scheumann started to introduce a myc-tag into the γc chain and could demonstrate that B cells and T cells modified with the myc-tagged transgene can be depleted *in vivo* and *in vitro*. Gene therapy with this molecule has recently led to the development of T cell leukemia in some patients due to retroviral integration; and trials have been discontinued until safety is ensured. A third adoptive therapy with an urgent need for a safeguard is the transfer of allogeneic T cells into patients that had received a hematopoietic stem cell transplant from the same donor. Though this treatment is highly effective against relapse and virus-induced lymph-proliferative diseases, a high incidence of severe, often lethal

GvHD requires a possibility to eliminate allo-reactive T cells in the patient. The modification of the lymphocytes with a membrane-anchored tag prior to transfer could allow treating GvHD while probably maintaining the anti-tumor effect.

Apart from acting as a safeguard, the introduction of a tag into the TCRα-chain offers the possibility of staining the molecule with a specific antibody, e.g. for FACS analysis without loss of functionality. As currently only very few monoclonal antibodies for different Vα variants are available this for the first time provides the means to differentially detect the transgenic TCRα-chain among the endogenous ones. Initial data from Simone Reuss in our laboratory show that a myc-tag and an HA-tag can be employed to discriminate between two different TCRα-chains of the same subfamily which has not been possible so far.

5 Abbreviations

7-AAD	7-amino-actinomycin D
aa	Amino acid
ADCC	Antibody-dependent cell-mediated cytotoxocity
APC	Allophycocyanin
ATCC	American Type Culture Collection
B6 mice	C57BL/6J mice
CAR	Chimeric antibody receptor
CDC	Complement-mediated cytotoxicity
CDR	Complementarity determining region
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
DED	Death effector domain
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E:T	Effector to target
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FasR	Fas receptor
FC	Flow cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat
FKBP	FK506 binding protein
γc chain	Cytokine receptor common gamma chain
GCV	Ganciclovir
GFP	Green fluorescent protein
GMP	Good manufacturing practice
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
HAMA	Human anti-mouse antibody
HSV-TK	Herpes Simplex Virus thymidine kinase
i.p.	Intraperitoneal
i.v.	Intravenously
IHC	Immunohistochemistry
IL-2	Interleukin-2

ITAM	Immunoreceptor tyrosine-based activation motif
LNGFR	Low-affinity nerve growth factor receptor
LTR	Long terminal repeat
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLV	Murine leukemia virus
NK cells	Natural killer cells
ova	Ovalbumin
P2A	2A element of porcine teschovirus
PBMC	Peripheral blood mononuclear cells
PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PRE	Posttranscriptional regulatory element
rhIL-2	recombinant human interleukin-2
RIP	Rat insulin promoter
RN	RetroNectin
RNAi	RNA interference
SD	Standard deviation
TAA	Tumor-associated antigen
TCR	T cell receptor
TIL	Tumor-infiltrating lymphocyte
TSA	Tumor-specific antigen
UV	Ultraviolet
wt	Wild-type
X-SCID	X-linked severe combined immuno-deficiency

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Statement

Hiermit erkläre ich gemäß der Promotionsordnung der Humboldt-Universität zu Berlin vom 01. Oktober 2002, dass ich

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